

# ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

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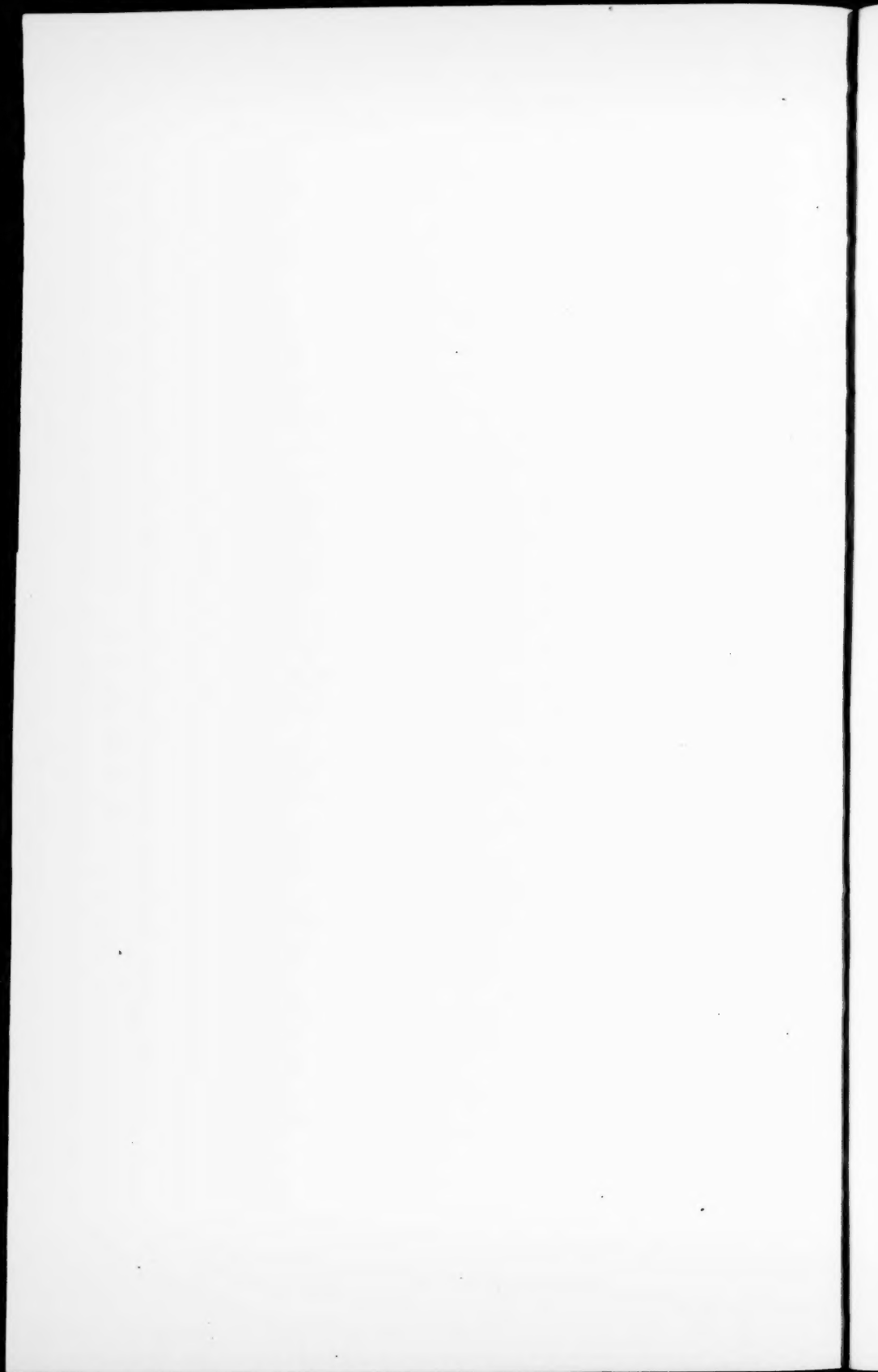


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## ANTIBODY-TRANSFORMING ACTIVITY

by

GRETA WASASTJERNA

(Received for publication October 17, 1955)

In certain cases of Rhesus-serum with albumin antibodies of type anti-D, Lewis and Chown demonstrated the presence of a so-called «hidden saline antibody» that agglutinates Rhesus-positive erythrocytes in the presence of a transforming serum. According to these workers transforming activity is not a characteristic of all sera, but appears in varying degree in about 10 per cent. In order to gain further insight into the nature of such «transforming activity» we carried out the following experiment:

In our investigation serum «T», which contains hidden saline antibodies, was a sensitized ORh-serum from which the  $\alpha$  and  $\beta$  agglutinins had been absorbed. After the absorption, an albumin-antibody titre of 1:712 was measured, while no saline antibody was demonstrable. This serum was diluted 1:4 in saline solution.

In a 5 mm. test tube equal parts of serum «T», a random serum and random ORh-positive erythrocytes in a 2 per cent saline solution were incubated for 45 minutes at 37° C. Microscopic analysis showed that agglutination had taken place in many cases, being in the majority of cases so faint, however, that interpretation became subjective. Since, apparently, the transforming activity is of highly varying intensity we decided to study further only those transforming sera which, when diluted with saline solution 1:8, still produce demonstrable agglutination. Six (6) such sera were obtained out of about 300.

Blood group determination of these six transforming sera was made with regard to the ABO and Rhesus systems and the total protein was determined. Five (5) sera which definitely did not produce agglutination were studied in a similar way. The following results were obtained:

A Transforming Sera	Blood Group	Total Protein per cent	Sedimentation Rate
1	ARh+	7.2	18
2	BRh—	7.5	
3	ARh+	8.4	
4	ORh+	10.2	
5	ORh+	6.0	
6	ARh+	8.4	
		mean: 7.95 per cent	
B Non-transforming Sera	Blood Group	Total Protein per cent	Sedimentation Rate
1	BRh+	9.0	9 117 10
2	BRh+	8.4	
3	ARh+	9.6	
4	ARh+	7.2	
5	ARh+	9.0	
		mean: 8.64 per cent	

In nephelometric (sulfosalicylic acid) protein determination, an error of  $\pm 2$  per cent is to be reckoned with.

No correlation with blood groups or protein content could be traced. Sedimentation rates were measured in isolated cases from both groups. A high sedimentation rate does not seem to be the factor governing the transforming activity.

We are able to confirm Lewis and Chown's observation on the occurrence of a »transforming activity». This activity is rarely (about 2 per cent) of any high order. There does not appear to be any correlation between it and the ABO and Rh systems or the total protein content of the serum.

#### REFERENCE

Nature, January 2, 1954 (M. LEWIS, BRUCE CHOWN).



## A HAEMOLYTIC TRANSFUSION REACTION DUE TO ANTI-Le<sup>a</sup>

by

ERIK E. ANTTINEN, H. R. NEVANLINNA, and TAPANI VAINIO

(Received for publication November 4, 1955)

Since the Lewis blood groups, thanks to investigations by Mourant (1) and Andresen (2) became known, Lewis antibodies are found more or less regularly in all blood grouping laboratories. It has not been possible to show conclusively that either transfusion immunization or pregnancy immunization are responsible for the development of Lewis antibodies. From the practical point of view, Lewis blood group factors have been considered weak antigens, assumed to cause no serious complications in connection with blood transfusion or pregnancy. The following report describes a case of haemolytic blood transfusion complication evidently due to Lewis antibodies.

*The Case.* — Mrs R., an office clerk of 27, had suffered for a prolonged period from menorrhagic discharge. She was admitted for treatment to a gynecological hospital. Myomata uteri were diagnosed as the cause of the bleeding. The gynecologic and obstetric history was otherwise normal. She had one healthy child of 2. Because of the severe anaemia due to the bleeding the patient was transfused in the hospital. In the course of the transfusion she felt pain in the low back and had chills. However, the transfusion was continued, and the patient received a total of approx. 600 ml of blood from two donors. Cross matching had been made twice as the result of the first investigation proved unclear. A few hours after the transfusion the patient began to vomit, and her urine became reddish. Icterus was observed and it increased during the next few days, likewise the nausea and vomiting. The daily urine output dropped to approx. half the normal. After two weeks, her condition continuously deteriorating, the patient was transferred to the First Medical Clinic of Helsinki University; diagnosis: Myomata uteri, Icterus haemolyticus.

In this hospital the patient was found to be in poor condition, she was very pale, slightly yellow, with large spots of itching eczema on the skin. No special cardiac and pulmonary findings were made. R.R. 110/70. The liver and the spleen were not palpably enlarged. Her daily output of urine was 400—600 ml; axillary temperature 38° C. Laboratory findings on admission are shown in Table 1. Uraemia, anaemia and, in addition, urinary infection were diagnosed.

TABLE I

Hb 37 per cent (Sahli)	Meulengracht 1 : 2
E 2.3	Stolte 2.1 ml
I 0.80	Bleeding time 1'20"
L 10.800	Clotting time 3'—5'30"
Lymphopaenia	Red cell osmotic fragility:
Thromboc 278,000	beginning haemolysis 0.54
Nonprotein nitrogen 165 mg/100 ml	complete haemolysis 0.24
Urine: alb. —, sugar —, sed.: plenty of leucocytes and gramnegative rods.	
Bile pigments: —	

Course of the disease: The patient's axillary temperature was 38—39° C. She was given fluid parenterally and streptomycin to control the urinary infection. The daily output of urine returned to normal in a couple of days. Nonprotein nitrogen decreased. Haemoglobin remained at approx. 37 (Sahli) for the first four days. After 11 days in the hospital the haemoglobin had dropped to 30 (Sahli), and a transfusion was considered necessary. A couple of suitable donors were found after numerous cross matching tests, but the specificity of the antibodies could not be determined. The transfusion of 800 cc of blood was followed by a fever reaction ad 40° C. No special findings were made in the urine. After a couple of days the treatment was repeated, again with a fever reaction ad 40.6° C. Again no signs of haemolysis were found. After the transfusions the patient's condition improved rapidly and her blood values rose (see Table II). The patient was discharged, convalescent, after 28 days.

TABLE II

	Days	1	4	7	11	14	16	18	22	26
Hb (Sahli) ....	%	37	37	—	30	39	47	—	56	67
Nonprot. N. ..	mg %		165	140	90	55	—	35	30	—
Alk. reserve ..	vol %			40						

## SEROLOGIC INVESTIGATIONS

We found Mrs R. to belong to blood group B Rh-negative (cde/cde) (C<sup>W</sup>—, MN, S—, Kell—, Le<sup>a</sup>-Le<sup>b</sup>+) No Rh antibodies were found on examination. One of the bloods causing the com-

plication was found to be Le<sup>a</sup> positive, of the group B Rh- (cde/cde) Le<sup>a</sup> + Le<sup>b</sup>—. The other donor was not traced. The patient's serum was found to contain an agglutinin reacting with 25 per cent of the 0 cells studied. Since the serum was tested with known red cells the presence of anti-Rh, anti-Kell, anti-Lutheran, anti-MNS or anti-P antibodies was excluded. Of the red cells at our disposal, the patient's serum agglutinated only those with a Lewis<sup>a</sup> antigen, best in albumin medium and with Coombs's technique. The reactions were most marked at + 37° C, whereas at room temperature and particularly at + 4° C non-specific agglutinins were also found. An additional frequency investigation showed that the patient's serum, with albumin and Coombs's technique, agglutinated 57, or 25.2 per cent, of 226 washed 0 cells. From these investigations, the patient's serum was considered to contain anti-Le<sup>a</sup> as a specific antibody, and in addition non-specific cold agglutinins<sup>1</sup>. A follow-up examination 3 years later gave a concurrent result.

#### DISCUSSION

The patient, whose medical history contained one normal delivery but no blood transfusions or blood injections, suffered from a severe haemolytic transfusion reaction. The child was compatible (B M Rh— Le<sup>a</sup>—Le<sup>b</sup> +). The transfusion was performed two years after delivery. The cause of the complication was traced back to an antibody of type anti-Lewis<sup>a</sup> in the patient's serum. Similar cases, though few, have been reported in the literature; in them, however, the reaction has been less severe (3), (4). The agglutinin found also revealed some features typical of Le<sup>a</sup> antibody, described by Stratton (6). For instance, it haemolysed cells *in vitro*, and in connection with a follow-up examination specificity to Coombs's reagent was found (5). This latter phenomenon may be due to incomplete cold agglutinins found in the serum. It is not possible to say anything definite of the development of the said agglutinin. Lewis antibodies, reported in the literature

<sup>1</sup> In addition, the serum in question was submitted to the Blood Group Reference Laboratory, Lister Institute. The result of the investigation carried out by Dr Dorothy Parker concurred with that of our investigation. We wish to take this opportunity of extending to her our best thanks.

in many contexts, are called spontaneous agglutinins; there is no conclusive evidence in the present case of an earlier sensibilisation against Le<sup>a</sup> antigen.

#### SUMMARY

A case of a transfusion reaction is reported in which anti-Le<sup>a</sup> antibody induced a severe haemolytic transfusion reaction. The anti-Le<sup>a</sup> agglutinin found in the patient's serum reacted with 25.2 per cent of the 0 cells from Finnish donors.

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  5. DACIE, J. V.: *Ibid.* 1953:166:36.
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## DETERMINATION OF THYROID ACTIVITY BY QUANTITATIVE MICRODENSITOMETRY USING $I^{131}$

by

LAURI SAXÉN, ERKKI SAXÉN and SULO TOIVONEN

(Received for publication November 8, 1955)

In the determination of thyroid activity two principal methods have been employed: chemical or physical measurement of the iodine content in the gland or in the body, and determination of the activity on the basis of the morphological changes in the gland. The most commonly used histological methods are the so-called d/n method (4, 5, 6) and histoquantitative linear measurement (11, 13, 14). When radioactive iodine is employed in studies on the iodine metabolism of the thyroid gland, the commonest and undoubtedly most accurate method is to use a Geiger counter in the determination of the iodine content. Another method, which hitherto has engaged less attention, utilizes quantitative autoradiography. The iodine stored in the thyroid is determined by counting the number of grains developing in a photoemulsion (7), or by densitometric methods (3, 8). In what follows a technique is described by which the writers have tried to combine the histoquantitative linear method with densitometric determination of an autoradiogram. In this way it is possible to estimate the degree of histological activity and the iodine content in one and the same gland.

### PROCEDURE

Larve of the South-African clawed toad *Xenopus laevis*, 5–10 cm. in length, were used as experimental animals. The classification of the material is described in connection with the results.

*Autoradiographic Technique.* — After preliminary experiments the same technique was invariably used in all experiments, the dose, exposure, development time and temperature being kept constant. The technique in question is as follows:

1. Four days' treatment in a solution of  $I^{131}$  ( $200 \mu\text{C}$  carrier-free  $I^{131}$  in 1000 ccm. of water +  $22^\circ\text{C}$ .).
2. 26 hours' fixation in Bouin's solution.
3. Washing in absolute alcohol, xylol and paraffin, 22 hours. (The various steps were performed in a «Histokine» apparatus.)
4. Embedding in paraffin, serial sectioning at  $10 \mu$ , removal of the paraffin, and drying (24 hours).
5. Preparations were covered with a celloidine membrane (a 0.4 per cent solution, one minute).
6. Preparations were placed on a film by the apposition method (Guilleminot Collodium 4 film; emulsion thickness 12 microns).
7. Exposure for 4 days.
8. Development for 5 minutes (Agfa «Final»,  $+18^\circ\text{C}$ .).
9. Fixation.

After drying, the autoradiograms were mounted in Canada balsam each on a glass slide with a coverslip (Fig. 1).

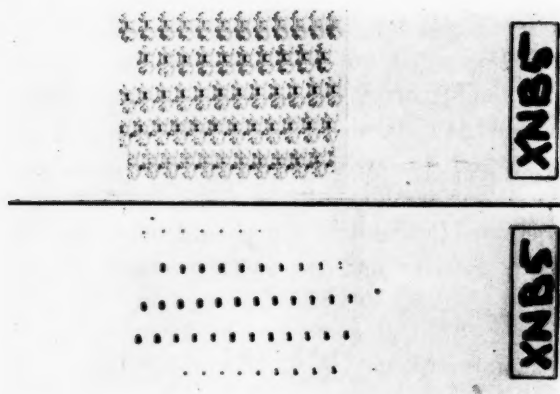
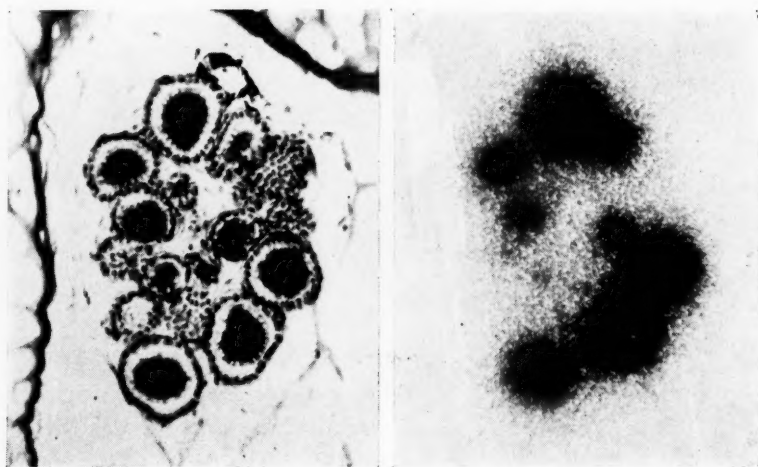


Fig. 1. — Serial sections from the thyroid gland of the toad *Xenopus laevis* and the corresponding autoradiograms mounted on a microscope slide. 1:1.



Figs. 2 and 3. — Photomicrographs showing the thyroid gland of the toad *Xenopus laevis* and the corresponding autoradiogram with iodine —  $^{131}$ .  $\times 200$ . (Magnifications of the preparations in Fig. 1.)

*Histoquantitative Methods.* — The degree of histological activity was determined by both the methods referred to in the introduction. The d/n relation (Lever) was determined in 24 follicles, four follicles in each gland of three animals being measured in each experiment ( $2 \times 3 \times 4 = 24$  follicles). The percentages of epithelium and colloid (Uotila) were calculated along three lines, measuring 20 cm. each, from three sections from each gland ( $2 \times 3 \times 3 \times 3 \times 20 = 1080$  cm.) using a microprojector with an objective  $20 \times$  and an ocular  $12 \times$ .

*Densitometric Methods.* — The density of the autoradiograms was measured by two different techniques. A micromethod proved suitable for determination of the autoradiograms of very small glands or large, single follicles, whilst a macromethod was found to be preferable in the study of larger glands.

*The Micromethod.* — In the phototube of a microscope was placed a photoelement at a fixed distance from the centre of the ocular image. The photoelement was coupled to a galvanometer. In order to maintain constant illumination a current strength regulator was placed between the light source of the microscope and the circuit. The apparatus employed is demonstrated in Fig. 4.



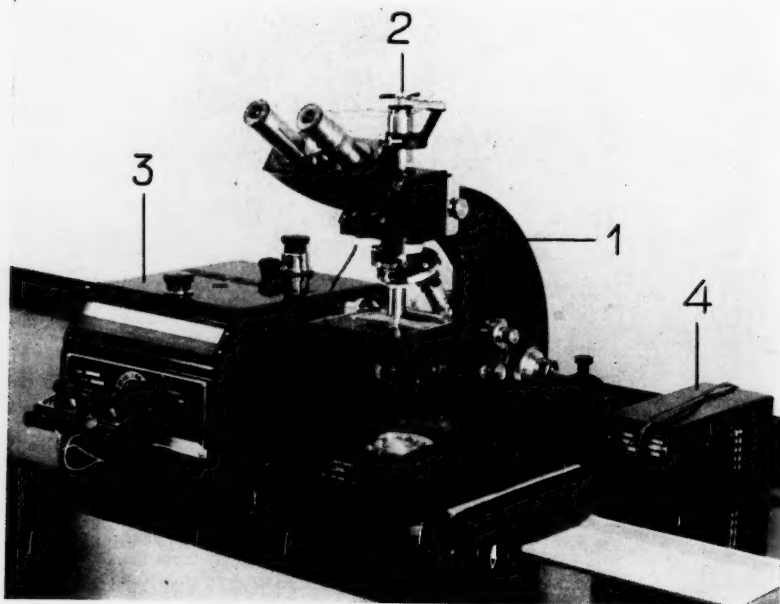


Fig. 4. — Apparatus employed in quantitative microdensitometry. 1. Microscope, Reichert «Zetopan» (objective  $40\times$ , ocular  $18\times$  photo). 2. Photoelement. 3. Multiflex galvanometer (Type MG2). 4. Current regulator. (The last three items manufactured by Dr. B. Lange, Berlin.)

Using this technique, density measurements are very rapid. The experimenter chooses the spot to be determined with the aid of a bitube, turns the latent image towards the phototube, and reads the result straight from the galvanometer.

From each series of sections from glands of  $250\text{--}400\ \mu$  thickness, the ten middlemost sections were selected for determination. The diameter of the field measured  $150\ \mu$  was about half that of the glands. The density of the autoradiograms was calculated as a percentage (D%) of the light background (0) of each slide. The results presented here are the means for both glands from three animals ( $2 \times 3 \times 10 = 60$  determinations).

Using this method the density measured corresponds to the total radiation of a certain area of the thyroid. However, owing to the structure of the thyroid, the dose of iodine, the time of treatment and other variables, the iodine is not evenly distributed over the whole area measured (3, 15). Hence only the really »active»



portion of the area measured must be considered when the final figures are compared. With regard to the present material it was found that the radioactive iodine was localized almost exclusively in the intrafollicular colloid. The relative amount of the colloid showed great variations in different groups of experimental animals, and thus the uncorrected density figures from different glands are not directly comparable. With accurate, quantitative radiography it should be possible to calculate the amount of radioactive substance in the tissue (2, 9). This is not, however, the purpose of the present, more practical method. The rather crude apposition technique here employed allows a high degree of scattered radiation, and the autoradiogram obtained does not correspond accurately to the real location of the iodine (Figs. 2 and 3). For this reason also, exact quantitative calculation is impossible. The density of an area to be determined has to be expressed in relation to the amount of the «active» tissue in this area, *viz.* in relation to the amount of colloid. The value thus obtained, which the present writers have called the «iodine value», is only a relative value, which expresses the iodine concentration in the intrafollicular colloid as a ratio density percentage/colloid percentage:

$$\text{I.V.} = \frac{D\%}{C\%}$$

*The Macromethod.* — The present experiments were performed on very small glands, with a diameter of only about 0.3 mm. In such cases a micromethod is almost indispensable. With a view to simplification of the micromethod described, a macroscopical densitometric procedure was also tried. This method might be employed when larger glands are studied. For this purpose the same autoradiograms were magnified 12.5  $\times$ . Magnification was obtained with a reproduction apparatus, and both exposure and development time were constant. These magnified autoradiograms were measured with an Autotype Major densitometer with an aperture of 1.85 mm., or about half that of the magnified autoradiogram of the gland. The densities are presented in the table as the inverse value of the mean densitometer reading.

#### RESULTS

Results obtained by the present methods are presented in Table 1; a more extensive investigation using the same method

TABLE 1

RESULTS OF HISTOQUANTITATIVE DETERMINATION OF THE THYROID GLANDS IN TWO GROUPS OF EXPERIMENTAL ANIMALS, NORMAL (1) AND NEOTHENIC (2), DENSITOMETRIC RESULTS OBTAINED ON THE  $^{131}\text{I}$ -AUTORADIOGRAMS OF THE SAME GLANDS AND THE "IODINE VALUE" (D%/C%) CALCULATED FROM THE LATTER. (ALL THE DIFFERENCES BETWEEN GROUPS 1 AND 2 OBSERVABLE IN THE TABLE HAVE BEEN STATISTICALLY ANALYZED AND FOUND HIGHLY SIGNIFICANT,  $P < 0.1$  PER CENT.)

	1	2
Epithelium percentage (Uotila) .....	39.3	15.3
Colloid percentage (C%) (Uotila) .....	54.2	74.4
D/n (Lever) .....	1.80	1.95
1/densitometer reading (macrosc.) .....	2.44	0.66
Density percentage (D%) (microsc.) .....	97.7	54.2
"Iodine value" (D%/C%) .....	1.80	0.73

is in preparation (10). The values listed in Table 1 were obtained on investigating the thyroid glands in two groups of experimental animals, which were simultaneously treated in the same iodine solution. The autoradiographic films were also simultaneously developed. Group 1 represents a largely normal thyroid, whilst group 2 consists of so-called neothenic larvae, the metamorphosis of which has been arrested with resulting marked atrophy of the thyroid (12).

It is seen in the table that the histological picture of the thyroid glands in group 2 is characterized by a high percentage of colloid, low epithelium, and a correspondingly high d/n value. The iodine concentration of the colloid is markedly lower in group 2 than in group 1, where the amount of colloid is relatively smaller.

#### DISCUSSION OF THE METHOD

There is no intention to advocate the substitution of the previous histoquantitative methods by the present one. In view of the fact that, in particular with regard to small glands, it is difficult to investigate the thyroid both histologically and with a Geiger counter, the present study was undertaken in an attempt to combine a purely histological determination, with a rough estimation of the capacity of the same gland to store iodine. In comparison with the calculation of grains referred to in the foregoing, this method is undoubtedly less accurate, but owing to its great rapidity it permits a large series of observations. Furthermore the total radiation of a relatively large portion of the thyroid

is observed at each determination, and in extensive series of observations the difference in activity between follicles of different size (7) does not seem to constitute a source of error. The larger the glands and autoradiograms to be determined, the larger is the area that can be used for determination, and the smaller is the number of observations that can be regarded as sufficient. The results presented here were obtained on glands with a diameter of only some 0.3 mm., which were thus very unsuitable for either histoquantitative linear measurement or densitometric methods. When larger glands are investigated, a lower microscopical magnification may be used, and when the diameter of the gland to be investigated is more than half a millimeter, the microscopical technique may be substituted by the more rapid macroscopical technique. With increasing size of the follicles it is also possible using the microscopical method to determine the density of the autoradiograms yielded by single follicles.

In the present paper, it is impossible to discuss in detail the numerous sources of error in autoradiography; with regard to this point the reader is referred to Odeblad's (9) and Boyd's (1) extensive monographs. As already emphasized, however, the method presented here is not used in an attempt to calculate the absolute amount of the isotope present in the tissues; it is used only for comparative studies. Thus the procedure gains in simplicity, and the most serious sources of error may be eliminated by maintaining as constant processing conditions as possible in all experiments with regard to dose, exposure, development, etc. Furthermore it should be pointed out that it is possible to compare the autoradiograms obtained only when the amounts of radiation to be measured coincides with the linear part of the sensitivity curve of the film employed; values falling outside this cannot be utilized. Notwithstanding the sources of error and limitations here discussed the present method may be used as a crude, but easy and rapid aid in the determination of the capacity of the thyroid to store iodine. Furthermore it also seems possible to apply this method to other tissues where the distribution of the isotope is regular and where the areas storing it may be determined by the histoquantitative linear method.

## SUMMARY

A method is presented which combines histoquantitative examination of the thyroid with determination of the amount of iodine stored by the same gland. Autoradiograms are obtained from the gland using an iodine isotope, and these are densitometrically measured either micro- or macroscopically. The iodine concentration of the colloid is expressed as a ratio, the »iodine value» = density percentage/colloid percentage.

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## ASSAY OF CYTOSTATICA (COLCEMID CIBA) IN THE BLOOD AND URINE OF CANCER PATIENTS BY A TISSUE CULTURE METHOD<sup>1</sup>

by

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The increasing number of preparations used in cancer chemotherapy necessitates a method for determination of their concentration in the blood and other body fluids. In the present investigation an effort has been made to use a tissue culture technique in this respect. The effect of Colcemid (CIBA) (8) and of the serum and urine from Colcemid-treated patients on the proliferation of HeLa cells has been studied.

### MATERIAL

The HeLa cells were originally obtained from the Tuskegee Institute of the Carver Foundation (by the courtesy of Dr. P. G. Brown) and had been propagated in our laboratory for 10 months.

The culture technique used and the counting procedure have been previously described (6).

The Colcemid used was obtained through the courtesy of the CIBA company. The ampullae contained 1 mg in 1 ml, representing a dilution of  $10^{-3}$ .

### PROCEDURE

*Determination of the Effect of Different Colcemid Concentrations.* — Colcemid was diluted in Hanks's solution and 0.1 ml of this

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

dilution was added to test tubes containing 0.3 ml of serum and 0.6 ml of Hanks's solution. Thus the final volume was 1 ml and the final dilution of Colcemid 10 times that of the initial one.

The effect of Colcemid on the proliferation of HeLa cells was clearcut and either complete arrest of the proliferation occurred or growth was very little, if at all, influenced (fig. 1). When a dilution series of  $10^{-7.5}$ ,  $10^{-7.6}$ , ---  $10^{-8.9}$ ,  $10^{-9.0}$  was used, it could be shown that the change from complete arrest of growth to slight growth occurred between the dilutions  $10^{-8.0}$  and  $10^{-8.1}$ . In the latter dilution the number of cell nuclei in the tube was the same after five days as after two days. The percentage of mitoses was also studied, and the results are presented in table 1.

TABLE 1

PERCENTAGE OF NORMAL NUCLEI, MITOSES AND DESTROYED NUCLEI IN DIFFERENT COLCEMID DILUTIONS AFTER TWO DAYS GROWTH

Dilution	Normal Nuclei	Mitoses			Destroyed Nuclei			
		Total	Arrested	Normal	Total	Percentage of		
						Pyknosis	Karyorrhexis	Karyolysis
per cent	per cent	per cent	per cent	per cent	per cent			
$10^{-7.5}$	24	20	19.6	0.4	56	(18)	(15)	(67)
$10^{-7.6}$	23	20	19.2	0.8	57	(31)	( 8)	(61)
$10^{-7.7}$	22	17	16.0	1.0	61	(29)	(12)	(59)
$10^{-7.8}$	33	11	7.7	3.4	56	(29)	(11)	(60)
$10^{-7.9}$	65	7	4.3	2.7	28	(70)	( 5)	(25)
$10^{-8.0}$	72	8	1.8	6.2	20	(66)	(15)	(19)
$10^{-8.1}$	83	7	0.6	6.4	10	(70)	(12)	(18)
$10^{-8.2}$	90	2	0.2	2.2	8	(63)	(14)	(23)
$10^{-8.3}$	90	4	0.1	3.9	6	(58)	(11)	(31)
$10^{-8.4}$	89	4	0.1	3.9	7	(55)	(10)	(35)

Each figure is the arithmetic mean of counts from three slides made from replicate tubes. On each slide 500 nuclei were counted.

In repeated experiments it could be shown that the change in the effect of Colcemid occurred within the same narrow range whether 60 per cent or 15 per cent serum was used. The size of the inoculum proved to be very important. If the inoculum was very

# EFFECT OF COLCEMID CONCENTRATION ON THE GROWTH OF HeLa CELLS

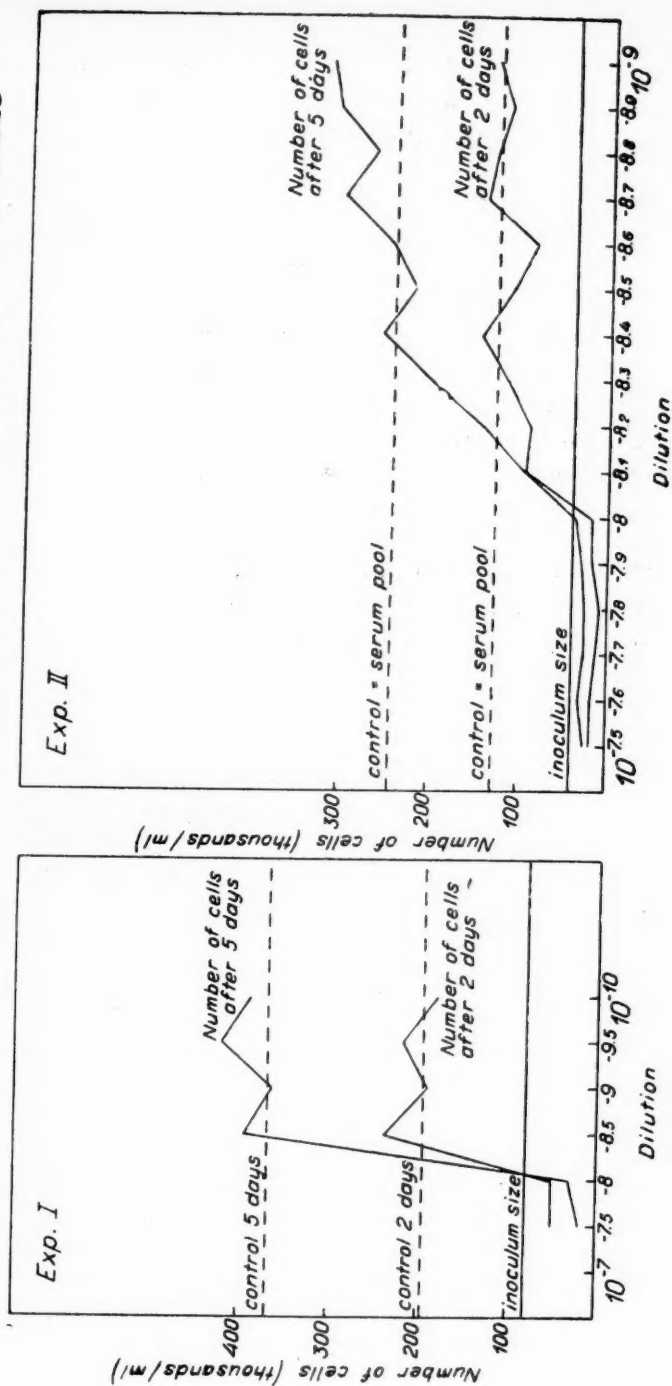


Fig. 1



small in size the results were difficult or even impossible to interpret. The inoculum size used in the present investigation varied from 40,000 to 80,000 cells per ml.

*Determination of the Inhibitory Effect of Patients' Serum and Urine after Colcemid Injection.* — As in earlier experiments a culture medium containing 30 per cent serum was used. Thus, when undiluted serum was used the Colcemid concentration in the test tube was 30 per cent of its concentration in the blood. Taking into consideration the results obtained with different Colcemid concentrations complete arrest of growth could be expected only when the serum contained 0.01 micro g Colcemid per ml. or more. When the serum to be studied was diluted, a serum pool was used as diluent in order to keep the serum content in the test tube constant.

The urine was diluted with pooled serum and this serum-urine mixture was used instead of serum in the tubes. The highest urine concentration used in the serum was 10 per cent. Thus the amount of Colcemid in the test tubes was at most only 3 per cent of that in the original urine.

*Determination of the Colcemid Content in the Blood and Urine.* — Since with the technique used the change in the effect of Colcemid on the proliferation of HeLa cells seems to occur within a very narrow range — between the dilutions  $10^{-8.0}$  and  $10^{-8.1}$  this phenomenon was used to determine the Colcemid content in blood and urine.

Dilution series were made up from the serum and urine to be studied and the effect of the different dilutions on the growth of HeLa cells was compared to that of known Colcemid concentrations. The nuclear count and/or the percentage of mitoses were used as criteria of growth.

*Example.* — A man, 60 years old, with pulmonary cancer. Pad. Carcinoma anapl. bronchi. Weight 50 kg. SR 90. White cell count 12,000.

The effect of the patient's serum on the proliferation of HeLa cells was studied in different dilutions before the intravenous injection of 5 mg Colcemid (CIBA) and 1, 3, 6 and 24 hours after it. The effect of the urine was studied before and 3, 6, 9 and 12 hours after the injection.

One hour after the injection the patient's undiluted serum was without effect in arresting the proliferation of HeLa cells. The arresting effect of



the urine was to be found in all samples taken after the Colcemid injection in the initial dilution  $10^{-1}$  but not at  $10^{-2}$ .

The test was repeated and samples of the blood were taken 15, 30 and 60 minutes after another injection of 5 mg Colcemid. (Between these two tests the patient had received 5 mg Colcemid and X-ray treatment on five consecutive days). Complete arrest of the growth of HeLa cells was observed with undiluted serum taken 15 minutes after the injection but not with that taken 30 minutes after. The dilution  $10^{-1}$  of the serum did not have any effect in any of the samples. The results are presented graphically in fig. 2.

#### DISCUSSION

The use of cultured cells for cancer chemotherapy screening has been employed in a number of investigations (1, 2, 3). The development of methods for growing large numbers of replicate cultures on surface substrates (4), and the measurement of proliferation in tissue culture by enumeration of cell nuclei (7) have further extended its use. Lettré and Lutze (5) have investigated the fate of colchicine in the animal body using as criterion its arresting effect on mitosis (Mitosestop) in heart fibroblast culture.

In the present study it could be shown that the tissue culture method can also be used in the determination of the growth-inhibiting effect of serum and urine from Colcemid-treated patients and that the Colcemid concentration can be nearly quantitatively determined in the body fluids by counting the cell nuclei.

Comparing the effect of different Colcemid concentrations on the growth of HeLa cells to that of the effect of the patient's serum and urine in different dilutions, a rapid disappearance of Colcemid from the serum and a prolonged excretion in the urine was observed.

It is to be anticipated that this method of quantitative determination can also be employed with other antineoplastic compounds which act directly on cells if their effect is as sharp as that of colcemid.

The various factors which might influence the results have been more extensively discussed in an earlier paper (6) concerning the effect of human sera on the growth of HeLa cells. Here some of the arguments are repeated.

The quantity and quality (e.g. the «age» of the cells) of the inoculum is of great importance. If the inoculum size is small, the results may be difficult or even impossible to interpret. Also, if the cells are «old» confused results may be obtained.

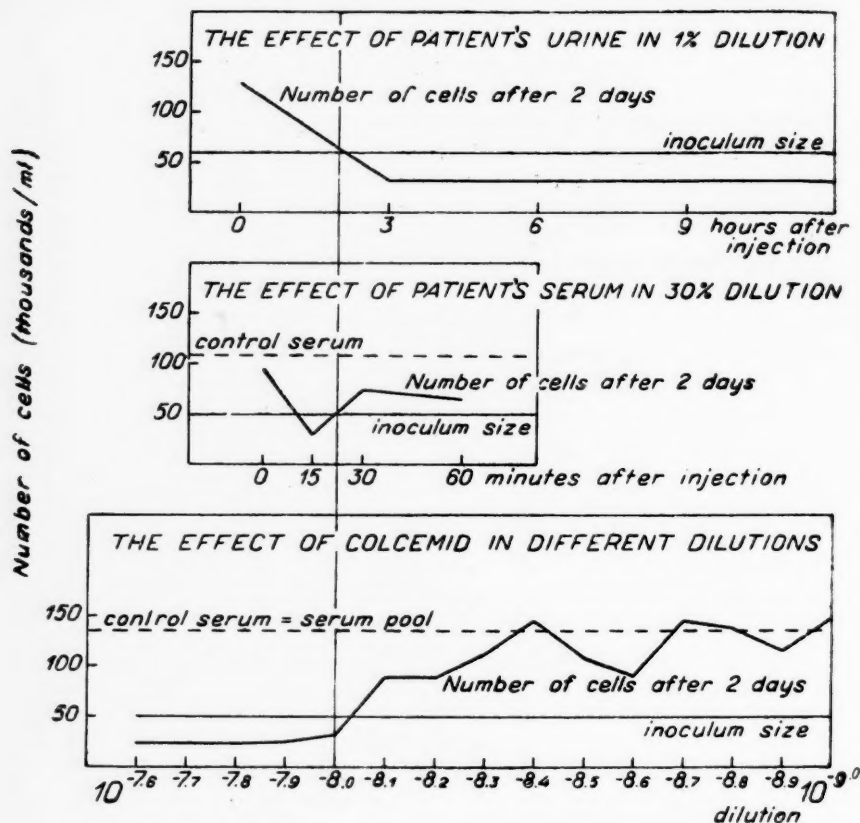


Fig. 2

With the technique used, the inoculum size varies even in the same experiment and in order to get reliable results triple determinations should be employed.

Although the counting of the percentage of mitoses and of nuclei showing degenerative changes gives more information on the growth process, it is not necessary in the assay of Colcemid in body fluids.

#### SUMMARY

A method for quantitative determination of the Colcemid content of the serum and other body fluids is presented. The method is based on the proliferation of HeLa cells in tissue culture.

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## EFFECT OF HUMAN SERA ON CULTURES OF HELA CELLS<sup>1</sup>

by

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It is possible to culture animal cells in synthetic media but the growth rate and the survival time of the cells is not as good as in the presence of serum or other fluids of animal origin (2, 4). In the early days of tissue culture Carrel and his associates, using chick embryo fibroblasts, studied the »growth-activating and growth-inhibiting principles of serum» and found that with increasing age of the chicks the serum loses its growth-promoting capacity to some degree (1). Later, several studies have been made with different types of cells and sera from patient's with different diseases (4, 5, 6, 10). The results, however, are fragmentary.

The object of the present study was to evaluate quantitatively the effect of different human sera, including different age groups and sera from untreated cancer patients, in increasing the number of HeLa cells (3) in culture. In addition mitoses were counted and morphological changes in the nuclei noted. According to the preliminary results hitherto obtained, there are differences in the growth rate of HeLa cells due to different sera. Surprisingly, some sera of old people have an equal or possibly better growth-promoting capacity than sera from middle-aged persons.

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

## METHODS

The HeLa cells used in this study were obtained by air directly from the Tuskegee Institute of the Carver Foundation Alabama, (by the courtesy of Dr. R. W. Brown and the National Foundation for Infantile Paralysis). The experiments described below were carried out with these cells during their 8—11 months of culture in our laboratory.

*The cultivation of the cells* was effected according to the method of Syverton et al. (9). The growth medium for the cells was composed of 30 per cent of pooled human adult serum, of 1—2 per cent of chicken embryo extract and of 68—69 per cent of Mixture 199 (4) manufactured by the pharmaceutical chemists «Orion», of Helsinki. The cells for the experiments were propagated in one liter Roux bottles containing 80 ml of the medium mentioned above. In transferring cells from bottle to bottle and to test tubes «Bacto trypsin» 1:250 was used as a 0.5 per cent solution. The inoculum size for bottles was at a 500,000—1,000,000 cell level and for test tubes (14 × 120 mm) at a 50,000 cell level. The total volume of cells and reagents in the test tubes was 1 ml. In dispensing the cells to tubes a magnetic stirrer and Cornwall pipetting unit was used. When sera were tested the «age» of bottle cultures was 2—3 days at transfer to test tubes. In view of preliminary observations on the effect of «age» of cells the age of the bottle cultures was considered important for obtaining similar growth rates in test tubes.

*Other Reagents.* — Hanks's balanced salt solution was made up by the pharmaceutical chemists «Orion». The sera for human serum pools were obtained through the courtesy of the Finnish Red Cross Blood Bank.

*Cleaning of Glassware.* — The method has been developed by A. Louhivuori, M. A., State Serum Institute, Helsinki. The hardness of the tap water in Helsinki is appr. 5 (D.H.). The cleaning solution used contained the following reagents per 10 liters: Na-hexametaphosphate 50 g, Rexopon L 25 g, (contains appr. 55 per cent of sodium salt of alkylarylsulfonate, trisodiumphosphate,

sodiumsulphate and Na-carboxymethylcellulose) and trisodium salt of ethylene diaminetetraacetic acid 50 g. The Rexopon L is manufactured by Helkavaara and Halme Co., Malmi, Finland. The glassware is immersed in the cleaning solution and heated to 100°C, after which the heating is stopped and the container left overnight. The glassware is rinsed 3—5 times with tap water and finally with pyrogen-free glass-distilled water.

*Collection of Serum Specimens.* — The blood specimens were mainly collected in vacuum venules after cleaning the skin with ether. The sera for comparative tests were obtained from different hospitals and from the staff of our laboratory. The experiments were usually done the day after the collection of the blood. The serum was separated from the blood clot by centrifugation after standing overnight at + 4°C. The sera were not inactivated.

*Cell Enumeration.* — When the cells were transferred from bottles to tubes, enumeration was done after the trypsinisation procedure (9) for reaching a suitable dilution of the cell suspension to be pipetted into the test tubes. The increase of the cells in the tubes after two and five days' incubation at 37°C was evaluated after the citric acid procedure (8).

All cell enumerations were done in a Buerker hemocytometer by calculating 100—400 cells. In the citric acid method some modifications were made to the procedure described by Sanford et al. (8). According to this method the nuclei were isolated by adding 7 ml of 0.1M citric acid to the tube used in the growth test. After incubation for one hour at 37°C one ml of 0.1M citric acid solution containing 0.1 per cent crystal violet was added, the tubes were shaken and the nuclei were centrifuged for 20 minutes at 1,800 r.p.m. Of the supernatant 8 ml was carefully withdrawn and the tube containing one ml of fluid and nuclei was vigorously shaken by hand. Thus throughout the procedure the nuclei were in the same test tube and the final volume was the same as the initial one.

Three tubes were virtually always used for each enumeration of cell nuclei. The counting of nuclei was done without knowledge of other experimental data.

The preparations for counting the mitoses and for studying the nuclear changes were stained by the Feulgen nuclear method.

## THE RESULTS

*The variation in Cell Counts of Inocula and of Final Counts.*— Although all possible means were employed to obtain an even distribution of cells in inocula, some variation was inevitable. Table 1 shows the counts of the same inoculum measured by the citric acid method in 12 different tubes and 6 tubes in another test.

TABLE 1  
VARIATION IN CELL COUNTS OF INOCULA

Exp. 1												
Number of tube	1	2	3	4	5	6	7	8	9	10	11	12
Number of cells (thousands/ml)	69	65	49	101	55	66	71	44	50	72	63	51
Exp. 2												
Number of tube	1	2	3	4	5	6						
Number of cells (thousands/ml)	111	107	91	86	105	99						

From the counts presented in Table 1 it can be concluded that considerable variation occurs in some series between the inocula in different tubes. One cause of variations seems to be the trypsinisation procedure. In our hands it has proved difficult to achieve dispersion of cell clumps to single cells. The degree of clumping of the cells varies in spite of trypsinisation. If counts after trypsinisation are compared with counts after citric acid treatment the former tends to give lower counts.

It was also observed that the protein content of the medium was of importance when the citric acid method was used. If citric acid was added to the cell suspension in Hanks's solution a high proportion of the nuclei were disintegrated and much lower counts were obtained. If the culture was «old» the cells seemed to be very fragile and the presence of proteins was of even greater influence. The counts in Hanks's solution could even be as low as  $\frac{1}{4}$  of that in 30 per cent serum. According to the experiments 3.75 per cent of



serum is enough to stabilize the counts in the citric acid method. Lower concentrations have not been examined.

To arrive at some idea of the reliability of the method twelve identically handled tubes after five days' incubation were treated with citric acid and the cell nuclei counted. Table 2 shows the results

TABLE 2  
VARIATION IN CELL COUNTS OF IDENTICALLY HANDLED TUBES; SERUM PERCENTAGE 30; FIVE DAYS' INCUBATION

Number of tube	1	2	3	4	5	6	7	8	9	10	11	12
Number of cells (thousands/ml)	241	216	245	192	278	198	267	242	216	254	284	225

The standard deviation as calculated from figures presented in tables 1 and 2 is in Exp. 1 15, in Exp. 2 10 and 30 in the Experiment shown in table 2. On the basis of these figures the table presented below has been calculated. It shows the 95 per cent confidence interval of the mean when the number of tubes used for calculating it varies from two to five.

Number of Tubes Used for Calculating the Mean	95 Per Cent Confidence Interval of the Mean		
	Table 1		Table 2
	Exp. 1	Exp. 2	
2	24	18	46
3	19	14	38
4	17	12	33
5	15	11	29

According to statistical analysis with three replicates, the 95 per cent significance level of the mean is  $\pm 38$  thousand cells/ml in the final counts. Series with inocula like those in Exp. 1 have to be rejected already at the start of the experiment.<sup>1</sup>

In the following experiments, as mentioned earlier, the counts presented are the arithmetical means of counts from three tubes, unless otherwise stated. Only values obtained in the same experiment with the «same» inoculum were compared.

<sup>1</sup> The authors are greatly indebted to Mr. E. Kaila Ph.D. for statistical treatment of the data.



*The Effect of Serum Concentration on Propagation of Cells.* — In most experiments HeLa cell proliferation was clearly dependent on the serum concentration. A typical experiment is illustrated in fig. 1.

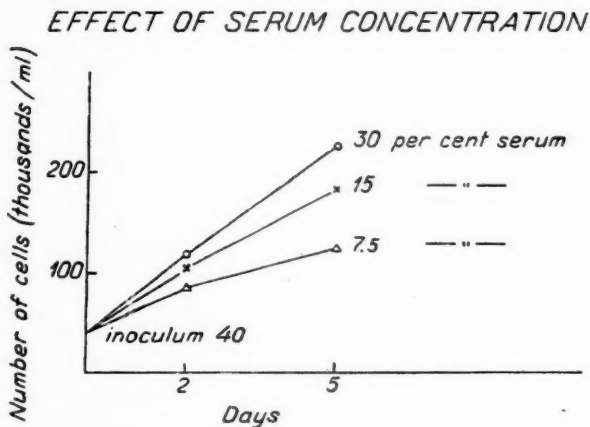


Fig. 1.

However a serum may, for some reason, artificial or natural, be inferior and the growth rate of cells is then not as much influenced by the serum concentration as with other sera. The growth-promoting capacity of placental cord serum seemed to be especially sensitive to serum concentration. (See Table 6 and Figs. 3 and 4. Serum 3 is placental cord serum).

*Effect of Serum Concentration on the Number of Mitoses.* — According to experience obtained, in higher serum concentrations, as is to be expected, the number of mitoses is higher than in lower ones. There seems however, to be, a tendency for the number of destroyed nuclei to increase with increasing counts of mitoses. It was also found that at 7.5, 15 and 60 per cent serum concentration the relative number of destroyed cells seems to be higher than at 30 per cent (Table 3). This would seem to imply that for the longest possible survival of cells a narrow serum concentration range might prove the most suitable.

*The Effect of Ice-box Storage on Sera.* — In our experience, the storage of serum for five days at  $+4^{\circ}\text{C}$  does not influence the growth-promoting capacity of sera. This does certainly not mean

TABLE 3

THE EFFECT OF THE SERUM CONCENTRATION ON THE PERCENTAGE OF MITOSES AND OF DESTROYED NUCLEI

Serum Concentration		After two Days		After Five Days	
		Mitoses	Destroyed Nuclei	Mitoses	Destroyed Nuclei
	per cent	per cent	per cent	per cent	per cent
Exp. 1	30	2.6	5.6	1.8	9.0
	15	1.4	8.2	1.4	13.0
	7.5	0.8	12.6	1.0	28.2
Exp. 2	60	4.2	2.2	1.4	14.6
	30	2.6	1.0	0.8	6.6
	15	2.2	1.6	0.4	21.8
Exp. 3	60	3.4	7.0		
	30	2.2	5.0		
	15	1.0	9.0		

Each figure is the arithmetic mean of counts from three slides made from replicate tubes. On each slide 500 nuclei were counted.

that possible exceptional properties of the serum do not change during ice-box storage.

*The results with sera of middle-aged persons* are presented in Table 4.

TABLE 4

THE RESULTS WITH FIVE SERA FROM THE MIDDLE-AGED GROUP.  
Inoculum size after trypsinisation 45,000 cells; Serum percentage 30

Serum	Age	Blood Group	Five-Day Counts (after Citric Acid Treatment) thousands/ml
E.S.	34	O	182
K.P.	37	O	223
M.M.	24	AB	223
A.T.	27	B	226
S.R.	23	A	214

Table 4 shows that no major differences are observed when sera of the middle-aged group are compared in the same experiment.

*Results with Sera from Different Age Groups.* — In Table 5 are presented the results of two experiments in which sera of old persons, middle-aged control serum, sera of one-year-olds, and of placental cords and of untreated cervix carcinoma patients are compared. The first experiment is presented graphically in fig. 2. In this graph the arithmetic means of the different groups are used.

Table 5 shows that when two- and five-day counts are used the sera of old persons are at least not inferior to the sera of other age groups or placental cords. The possibility that some sera of old persons are very good in promoting the growth of HeLa cells deserves further study.

The results indicate that the sera from untreated cervix carcinoma patients do not differ greatly from other sera, although

TABLE 5

THE RESULTS WITH SERA FROM DIFFERENT AGE GROUPS.

Inoculum size in experiment 1 45,000/ml and in exp. 2 57,000/ml after trypsinisation

Age years	2-day Count Thousands/ml	5-day Count Thousands/ml	
Experiment 1			
80	244	365	(E.S. in Table 3)
78	218	278	
75	314	409	
34	207	254	
1	204	324	
1	187	214	
Cord 1	251	389	
Cord 2	249	242	
Experiment 2			
80	392	454	The same as in exp. 1
75	268	407	» » » » » 1
25	203	373	
Cord 3	265	423	
Cord 4	262	no data	
Carcinoma	310	379	
»	205	220	
»	262	367	

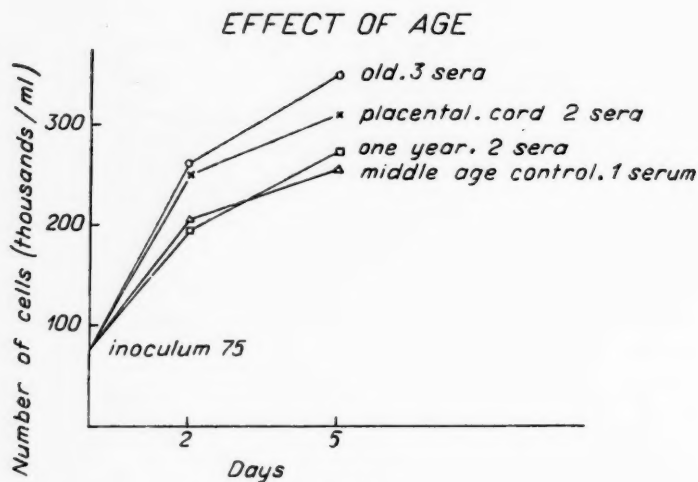


Fig. 2.

the percentage of mitoses seemed to be slightly greater and one carcinoma serum was inferior as compared with the others. However such «inferior» sera are occasionally found among «normal» persons also. So far, however, we have not found any «toxic» sera which do not promote the growth of HeLa cells.

Finally one experiment is presented in detail, the counts for each tube being given separately. In this experiment counts were compared with a pool composed of equal parts of the three sera in question. The results are presented in Table 6 and figs. 3 and 4.

The results show that serum No. 1 is inferior to the others used in the experiment. The inferior serum seems to have determined the behaviour of the pool at the 30 per cent serum concentration, although the data are invalidated by the loss of triplicate counts at 30% serum concentration and five days. Table 6 also gives some idea of the reliability of the counts. The variations between the triplicate cell counts are in general small.

TABLE 6

COMPARISON OF THE EFFECT OF SERUM POOL WITH THE EFFECT OF ITS COMPONENT SERA. INOCULUM SIZE 30,000/ML AFTER TRYPSINISATION. THE FIGURES REPRESENT THE NUMBER OF NUCLEI IN THOUSANDS/ML

	30 % Serum		15 % Serum		7.5 % Serum	
	2 Days	5 Days	2 Days	5 Days	2 Days	5 Days
Serum 1	63	179	56	123	52	77
	61	181	57	92	38	80
	59	170	58	85	58	53
Serum 2	157	289	117	268	82	162
	174	367	128	253	68	149
	159	313	no data	262	96	159
Serum 3	121	357	97	216	49	—
	130	354	95	186	55	70
	112	381	77	167	58	62
Pool	80	163	84	206	78	111
	91	no data	106	202	85	143
	no data	"	116	148	91	123

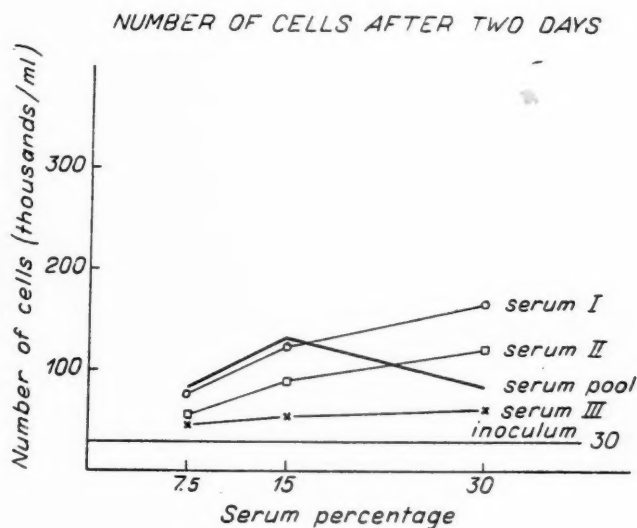


Fig. 3.

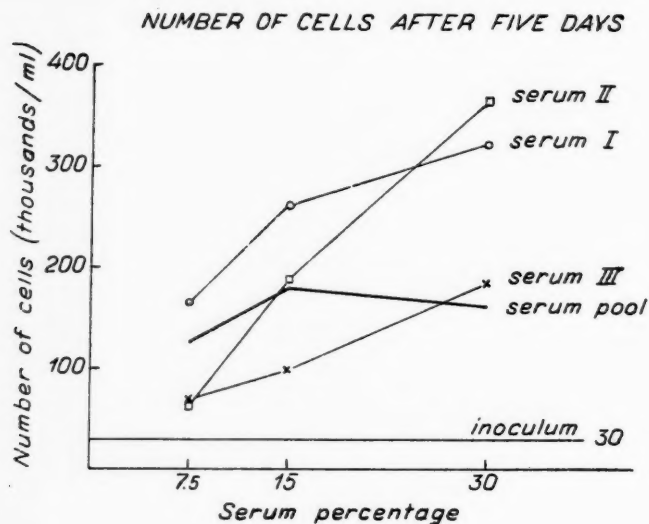


Fig. 4.

## DISCUSSION

Practically all media which promote abundant proliferation and long survival time of cells contain serum or some other fluid of animal origin. This serves to emphasize the importance of investigations aimed at evaluating the growth-promoting capacity of different sera. In some earlier investigations differences, between sera have also been found (5, 6, 7). Significant comparison of quantitative data, however, is a difficult matter with tissue cultures. When investigating the effect of sera the following considerations, at least, are of importance: The sera have to be collected by the same method and the time of storage before testing should be the same. The need for quantitatively and also qualitatively («age») equal inoculums is obvious. The number of tubes required for reliable comparison depends on the inoculum and final count variations. The enumeration of nuclei gives, however, only an incomplete picture of the cell growth if not also the mitose rate and the nuclear changes are taken into consideration.

In the present study not all the requirements mentioned above were completely fulfilled. The data presented, however, tend to indicate that there are differences between different sera. The

differences may be overlooked if the sera are not tested in different dilutions.

The possibility that some sera of old persons are very good in promoting the growth of carcinoma cells is interesting. The effect of sera from cancer patients deserves further study.

#### SUMMARY

Sera from different age groups and untreated cervix carcinoma patients have been compared in their capacity to promote the growth of HeLa cells. Attention is directed to the good growth-promoting capacity of some sera from old persons.

The factors influencing the reliability of the cell counts used in comparing the sera are discussed.

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## CYTOLOGY OF THE ANTERIOR PITUITARY AND THYROID FUNCTION

A STUDY OF DELAYED METAMORPHOSIS AND NEOTENY

by

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One of us has previously described a case of partial neoteny in the South African clawed toad (37, 38) accompanied by marked atrophy of the thyroid gland of the tadpoles. The investigations to be presented here are a continuation of this work. Furthermore, complementary studies were performed on a new neotenic strain, using radioactive iodine. The cytology of the anterior pituitary was investigated in the different groups of experimental animals.

### INTRODUCTION

In 1912 Gudernatsch showed that metamorphosis was accelerated in frog tadpoles fed with thyroid gland, and a couple of years later Adler (1) was able to show that thyroid function was dependent on the anterior pituitary, destruction of the latter resulting in atrophy of the thyroid gland and failure of metamorphosis. Thus it was convincingly proved that the anterior pituitary-thyroid system influences the metamorphosis of the frog, and simultaneously a significant biological criterion of thyroid activity was discovered, which »... still remains a most delicate test for the bioassay of thyroid material» (25). In view of these results it is natu-



ral that in subsequent investigations into the causes of disturbed or arrested metamorphosis attention was principally centered on thyroid function and its regulation.

In the amphibians metamorphosis may be arrested in various ways: In some species metamorphosis never takes place, but the animals nonetheless attain sexual maturity — absolute neoteny (32); some other species metamorphose under advantageous conditions whilst otherwise they remain at the larval stage, but in either case the animals attain sexual maturity — total neoteny (23). A third group consists of animals normally metamorphosing, though under adverse conditions sometimes remaining as abnormal, undeveloped larvae, which do not attain sexual maturity — partial neoteny (23). Without entering on a detailed discussion of the causes of these phenomena (illumination, temperature, nutrition, etc.), a brief presentation of some earlier observations concerning the anterior pituitary-thyroid system of neotenic individuals seems justified (for details the reader is referred to 24, 37, 36). Swingle (33, 34) found that the thyroid gland of the axolotl (total neoteny) contained thyroxine in abundance; nonetheless a preparation made of such a gland did not induce metamorphosis when injected into another animal. Hence this author concluded that the real cause of neoteny in the axolotl must be decreased sensitivity of the tissues to thyroxine and not a disturbance of thyroid function. In papers dealing with the same species the view has also been advanced, however, that the primary cause is hypophyseal, and that absolute neoteny, by contrast, is due to other causes, *e.g.* decreased tissue sensitivity (24). Many different theories have also been put forward with regard to the endocrine causative mechanism in partial neoteny, which is at present our main concern. Ingram (21) held that partial neoteny is probably caused by a decrease in hypophyseal thyrotropin secretion. Later this view has been supported by several authors (17, 9, 3, 2 and others). It has also been suggested that the thyroid gland itself is responsible for the condition in question, its capacity either to produce or to secrete thyroxine being disturbed (4, 39). Furthermore, a decreased sensitivity to thyroxine in the peripheral tissues has naturally been referred to as a third possibility, in particular with regard to the neoteny due to exceedingly low temperatures (20, 16, 11).

## MATERIAL AND METHODS

*Material.* — All investigations were performed on tadpoles and adult individuals of one and the same species, the South African clawed toad (*Xenopus laevis* Daudin), reared in our laboratory. Some tadpoles of several strains developed into neotenic individuals, their metamorphosis coming spontaneously and irreversibly to a standstill. Since in these cases the temperature (about  $+22^{\circ}\text{C}.$ ) and illumination had been normal during rearing, these environmental factors cannot have caused the neoteny. Furthermore, the individuals in question differed from normal tadpoles of the same developmental stage inasmuch as they were larger and stronger in build. In what follows they will be referred to as »neotenic». Some tadpoles were reared in a colder environment than normal ( $+16$ — $+18^{\circ}\text{C}.$ ), and in the dusk. Metamorphosis was arrested in these cases, too, but always reversibly; when the animals were transferred to a higher temperature, metamorphosis progressed normally. In this group, the growth of the tadpoles was also arrested, and their build was slender, in contrast to that of the neotenic. They will here be called »delayed». The normal control material includes tadpoles and adult individuals, either attaining the stage of development to be investigated or completely metamorphosing within a normal time (see Normal Table). The control animals were reared at about  $+22^{\circ}\text{C}.$  and were fed chiefly with dry, powdered liver as suggested earlier (26). As far as possible tadpoles of the same developmental stage were selected for investigation (Stages 54—56 of the Hubrecht Laboratory). It may be mentioned that we have previously (31) found that thyroid activity begins before this stage, at stage 50—51. The entire material is presented in Table 1.

*Methods.* — The hypothalamus, the hypophysis and the thyroid gland of all animals were histologically investigated. The hypothalamus and the hypophysis were fixed in normal formol-alcohol for 24 hours, after which the brains were dissected out. After ordinary dehydration the brains were mounted in paraffin and sectioned at  $5\ \mu$  in serial transverse sections. Staining was performed by Heidenhain's azan method, by microscopic differentiation. Furthermore Pearse's (27) trichrome-PAS method was employed after Helly's fixation for six hours. The thyroid glands

TABLE 1

MATERIAL USED IN THE INVESTIGATION (AGE AND STAGE OF DEVELOPMENT ACCORDING TO THE NORMAL TABLE OF HUBRECHT LABORATORY; LENGTH, AND NUMBER OF INDIVIDUALS INVESTIGATED)

	Age (Weeks after Hatching)	Stage (Hubrecht Laboratory)	Length mm	No. of Specimens
Normal .....	6	54—56	50	10
Normal .....	24	adult form	—	5
Delayed .....	12	54—56	50	10
Delayed .....	24	»	70	10
Neotenic .....	12	»	80	2
Neotenic .....	24	»	100	12
Neotenic .....	32	»	120	2

were fixed in Carnoy's or Bouin's solution, serial sections were made in the transverse plane at 10  $\mu$ , and these were stained by Heidenhain's azan.

In the investigations using the iodine isotope,  $I^{131}$ , living tadpoles were kept for four days in an iodine solution, after which autoradiograms were made of the thyroid sections. The density of the autoradiograms, which were thus made under standard conditions, was measured microdensitometrically; the iodine concentration of each gland is expressed as its «iodine value», which is only a comparative value (density percentage/colloid percentage). This method is described in detail elsewhere (30). Each iodine value indicated in the table is the result of 60 determinations.

The histological activity of the glands was determined by a histoquantitative linear method (40). Each final value for the percentages of colloid and epithelium is based on the determination of a total length of 1080 cm.

## RESULTS

*The Thyroid.* — In addition to the histoquantitative determination, the approximate size of the glands was determined by measuring the craniocaudal diameter of both glands in three animals in each group. These values and the ratio of the diameter to the body length are presented in Table 2.

TABLE 2

THE CRANIOCAUDAL DIAMETER OF THE THYROID GLAND IN THE VARIOUS GROUPS OF EXPERIMENTAL ANIMALS AND THE RATIO DIAMETER  $\mu$ /BODY LENGTH MM

	Diameter of Thyroid Gland (Microns)	Diameter in Microns Body Length in mm
Delayed 6 months .....	425	6.1
Delayed 3 » .....	290	5.8
Normal 1.5 » .....	210	4.2
Neotenic 6 » .....	290	2.9
Neotenic 8 » .....	310	2.6

It is clearly seen from the table that in the neotenic group the thyroid gland is atrophic with regard to size — it being only about 50 per cent larger than that of normal tadpoles in spite of the much greater size of the animals. The thyroid of the delayed tadpoles, on the other hand, is larger than normal although the animals are only slightly more than normal size, the diameter of the gland being about twice the normal length.

The results of the histoquantitative measurements, and the iodine values, are presented in Table 3. (In the group »Neotenic

TABLE 3

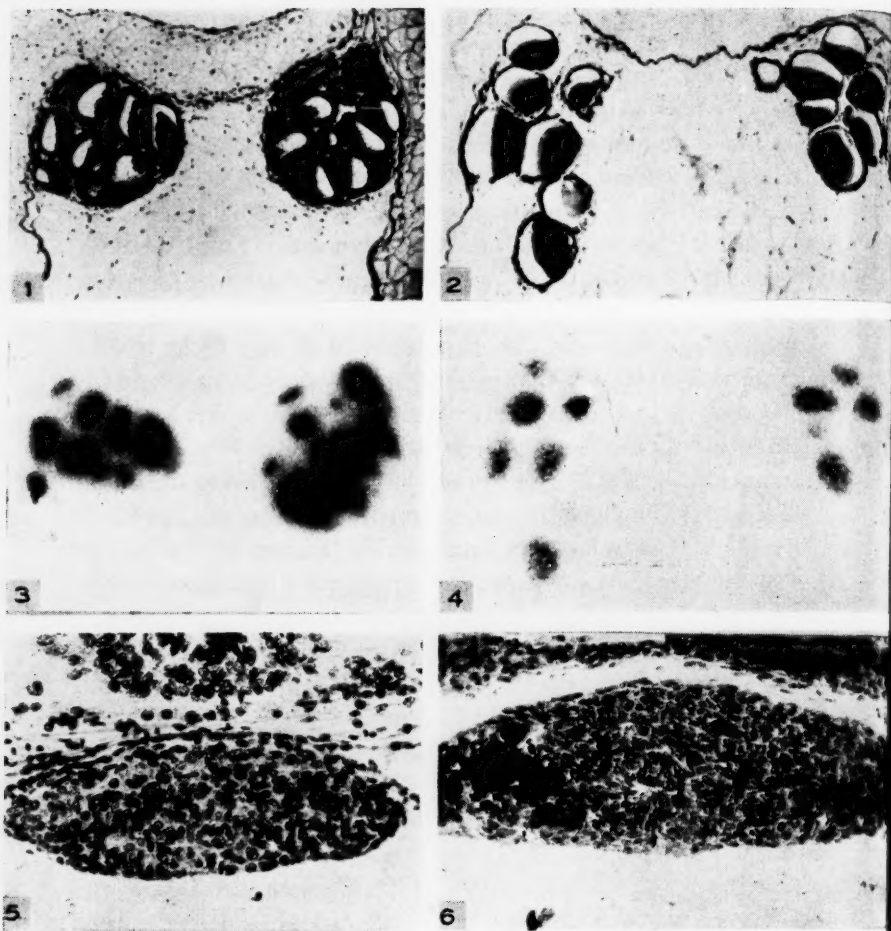
THE EPITHELIUM PERCENTAGE AND THE COLLOID PERCENTAGE IN THE THYROID GLANDS OF THE NORMAL, DELAYED, AND NEOTENIC TADPOLES, AND THE DENSITY OF THE AUTORADIOGRAMS OF THE SAME GLANDS. THE IODINE VALUE<sup>o</sup>, I.V., EXPRESSES THE RATIO DENSITY PERCENTAGE/COLLOID PERCENTAGE

	Epithelium Percentage E %		Colloid Percentage C %		Density Percentage D %		Iodine Value <sup>o</sup> I.V.
Delayed 6 months ..	41.6		46.9		92.2		2.02
	44.8	45.4	48.0	45.2	91.4	91.5	
	49.9		40.7		90.8		
	40.8		51.0		97.9		
Delayed 3 months ..	35.0	39.3	60.5	54.2	97.6	97.7	1.80
	43.2		51.0		97.9		
	32.5		56.7		88.5		
	39.4	35.4	53.5	56.3	92.5	91.0	
Normal 1.5 months ..	34.2		58.7		92.1		1.62
	28.2		64.0		—		
	29.0	27.4	60.3	67.1	—	—	
	26.0		70.1		—		
Neotenic 6 months ..	15.9		74.3		57.5		—
	14.9	15.3	79.1	74.4	50.8	54.2	
Neotenic 8 months ..							0.73

5 months» the iodine value was not calculated because the exposure time had been too long.) The table confirms the conclusions that could be drawn from the variations in size of the thyroid glands. In the neotenic tadpoles the thyroids display a high degree of atrophy, reflected in the low percentage of epithelium and the high percentage of colloid, whilst in the group »delayed» the glands show marked hypertrophy as compared with normal glands. In this group, the percentage of epithelium is increased and the relative amount of colloid is decreased. Marked differences are also discernible with regard to the capacity of the glands to store iodine. In the neotenic tadpoles the thyroid retains very little iodine, whereas the amount encountered in the glands of the delayed tadpoles is somewhat greater than normal.

*Anterior Pituitary.* — The cytological descriptions of the cells of the anterior pituitary to be presented in what follows are based on the two staining methods mentioned above, *i.e.* Heidenhain's azan and the trichrome-PAS method. The normal hypophysis of the species here concerned has previously been thoroughly investigated by the use of these methods (12, 13, 5, 6). The hypophyses of both the abnormal groups will here be described in relation to equally stained hypophyses of normal tadpoles and adults. In both groups, neotenic and delayed, the changes were so typical and uniform, irrespective of the age of the tadpoles, that each group will be treated as a whole.

*Delayed* (3 and 6 months). The anterior pituitary is rich in cells, which are roundish and contain little cytoplasm. With regard to structure it resembles the hypophysis of normal tadpoles of the same stage. However, the basophilic cells contain more cytoplasm than those of normal tadpoles and it is also possible with trichrome-PAS staining to demonstrate a stronger polysaccharide reaction in the area of basophilic cells than is the case with normal tadpoles. The basophilic cells are larger and more numerous. Their nuclei are round, on average  $4.5 \times 5 \mu$ ; the cells are irregular in shape and measure about  $7 \times 11 \mu$ . One or two nucleoli are clearly visible in the nuclei. Vacuolization and granulation are not observable in the cytoplasm, but the latter stains intensely blue. Faintly acidophilic cells are only encountered in the dorsal part, adjacent to the neurohypophysis, just as in the case of the hypophyses of normal tadpoles. The nuclei of these cells are fusiform and measure on



Figs. 1—6. — Photomicrographs showing the thyroid glands, the corresponding autoradiograms, and the transverse sections of the anterior pituitary in a delayed (Figs. 1, 2, 3) and a neotenic (Figs. 2, 4, 6) six months old tadpole of *Xenopus laevis*.

average  $3.5 \times 6.5 \mu$ ; the cells are poor in cytoplasm, and the red zone observable with azan staining consists mostly of nuclei. No chromophobic, or faintly basophilic, cells were encountered.

**Neotenic** (3, 6 and 8 months). The cells of the anterior pituitary are large, obviously larger than in the previous group and in normal tadpoles; they vary in shape. The sinuses are extensive, and with regard to shape and general structure the hypophysis



most closely corresponds to that of a 6-month-old, adult individual. In the area of the basophilic cells (in the ventral part of the central and caudal portion), the majority of the cells stain very faintly, however. With azan staining these cells become faintly bluish-grey, and they are PAS-negative. Their nuclei measure on average  $4 \times 6.5 \mu$ ; the cells are elongated, and often irregular, measuring about  $5 \times 10 \mu$ . Cells with two nuclei are occasionally seen. No granulation or vacuoles are clearly discernible in the cells. Red-staining, acidophilic cells are present in abundance, in particular in the rostral part. These cells are large and rich in cytoplasm, with round nuclei measuring about  $5 \times 6 \mu$ . The cells are irregular, but well-defined; they measure about  $7 \times 18 \mu$ . The cytoplasm is homogeneous and stains intensely. Some cells contain deeply staining vacuoles, varying in size from 2 to 6  $\mu$  in diameter.

When azan staining was employed, cells staining intensely blue, *i.e.* clearly basophilic cells, were rarely encountered. They were never observed in 3-month-old neotenic tadpoles, and only occasionally in 6- and 8-month-old tadpoles. The size of their nuclei was the same as in normal and delayed individuals, *i.e.* about  $4.5 \times 5 \mu$ , but they were poorer in cytoplasm.

With trichrome-PAS staining, PAS-positive material, corresponding to the basophilic elements, was encountered in only a few cells. Typical acidophils, staining intensely yellow with orange G, were encountered in abundance.

#### DISCUSSION

On evaluation of the results it must be presumed that in both groups, neotenic and delayed, the thyroid function is disturbed in some way. This presumption is supported by a large number of previous findings, summarized by Etkin (11) as follows: »It may be concluded on the basis of the evidence summarized in this section that the thyroid through its hormone is the primary determinant of metamorphic transformation in amphibians.» The present attempt to disclose the mechanism of hypothyreosis in the two groups of experimental animals is based on three different criteria: 1. the histological picture of the hypothalamus and the hypophysis, 2. the degree of histological activity of the thyroid gland, 3. the capacity of the thyroid gland to store iodine.

*Anterior Pituitary.* — It should be borne in mind that in spite of the large number of relevant investigations there is no consensus of opinion with regard to the question of which cells in the hypophysis secrete thyrotropic hormone. Hoskins and Hoskins (18), and Kerr (22), were inclined to ascribe thyrotropin secretion in the amphibians to the acidophilic cells of the hypophysis, and Willier (42) also stated: »Although it is difficult to relate specific cells to specific secretions, the body of evidence suggests that the acidophils may be associated with the production of thyrotropic hormone . . . » Most investigators, however, have assumed that it is the basophilic cells of the anterior pituitary that are the source of the thyrotropic hormone (7, 12, 13, 5, 6). According to Pearse (27, 28), the fact that the basophils stain by the polysaccharide method constitutes evidence that the mucoprotein hormones of the hypophysis (FSH, TSH and LH) are produced by these cells. Furthermore, in the mammals these cells are of two types, one producing gonadotropin and another producing thyrotropin (29, 43). The present results clearly showed that it is the basophils that stain by the polysaccharide reaction (PAS), since the former were invariably found in the same, fairly well-demarcated area as the PAS-positive cells. In what follows, a twofold distinction will therefore be made between acidophilic or PAS-negative, and basophilic or PAS-positive cells.

In contrast to the findings of some previous investigators (*e.g.* 17), the present results revealed marked differences in the cytology of the hypophyses of neotenic and normal tadpoles. When endeavouring to interpret these differences we are confronted by a peculiar difficulty, however. Is the presence of a high degree of granulation in a certain type of cell a sign of enhanced synthesis and secretion of hormone, or is the absence of granulation, by contrast, ascribable to intensified secretion? The latter view has been supported, for instance, by Cordier (6), and by Desclin (10), who wrote: »The cytoplasmic granulations which are usually thought to be hormonal precursors, increase during rest from secretion and diminish during excretory activity». Gasche (12, 13) investigated the effect of thyroidectomy and thiouracil on the anterior pituitary of *Xenopus* larvae; in both cases the histological picture was identical with that of the neotenic tadpoles described above. Since the thyroid hormone level was reduced in both cases, Gasche concluded that



the light, sparsely granulated »transformierte basophile Zellen» observed by him were exceedingly active, thyrotropic hormone-secreting cells. D'Angelo (8) also advocated the view that these well-known »thyroidectomy cells» are derived from basophils, and that their PAS-negativity »may indicate hypersecretion of TSH without storage.» On the basis of these investigations it seems likely that the picture displayed by the neotenic tadpoles, discussed above, is ascribable to intense secretion of thyrotropin. The highly granulated basophils of the delayed tadpoles, on the other hand, which, moreover, yield a strong PAS reaction, would thus be a manifestation of a decreased or perhaps entirely arrested thyrotropin secretion. It should be borne in mind, however, that the histological findings in question apparently represent a persisting condition, and that, by contrast, a weak granulation may be a sign of atrophy and decreased TSH secretion resulting from protracted hypofunction.

*The Thyroid.* — By the histoquantitative linear measurement method (40) employed it is possible to assess the degree of histological activity of the gland, but the level of the secretory activity of the thyroid cannot be determined on this basis, as has also been found in numerous experiments performed with antithyroid substances. On the other hand, the histological picture is related to TSH stimulation (35, 41 and others). In the neotenic tadpoles the thyroid gland was markedly atrophic with regard to both size and structure, whereas in the delayed tadpoles a gradually increasing hypertrophy was observable during development. In addition to the histological measurements, the capacity of the thyroid to store iodine was investigated, but these results, too, reflect the iodine metabolism of the gland only in part, and it is not possible to assess the level of the secretion of thyroid hormone on this basis either. The iodine values obtained must therefore be related to the above-mentioned histological observations, and these results must be evaluated together. From this standpoint the low iodine values of the neotenic tadpoles do not seem to be a sign of rapid iodine metabolism and a consequent scantiness in the storage of iodine, since the atrophy of the gland is evidence in favour of a decreased activity. The secretion of thyroid hormone must also be decreased, therefore, and the neoteny must be ascribed to a decrease in the production of hormone and not to decreased tissue sensitivity. The mechanism of hypothyreosis in this group will be discussed later.

In the group »delayed», a rise in the iodine value was observed in addition to marked hyperplasia. It is not possible definitely to state whether increased secretion of thyroid hormone is also involved, or whether secretion is disturbed, as it is, for instance, when certain antithyroid substances are administered.

As mentioned in the introduction, the primary disturbance in arrested metamorphosis may theoretically be of three kinds. Firstly, the thyrotropin system regulating thyroid function may be inhibited; secondly, the synthesis or secretion of the thyroid hormone may be disturbed, and thirdly, it may be suggested that the sensitivity of the peripheral tissues to the thyroid hormone is decreased. It should at once be stated that on the basis of the results presented here, it is not possible to conclude with certainty the mechanism of the disturbance in either of the groups under investigation, neotenic and delayed, but it is certain that the mechanism is not the same in the two.

In the light of previous investigations (12, 13, 5, 6, 8), the histological picture of the anterior pituitary here described seems to be indicative of increased secretion of TSH in the neotenic tadpoles. The atrophy of the thyroid gland and the low iodine value must then be ascribed to inability of the gland to react to TSH stimulation. This would lead to a disturbance of the hormone synthesis, a decrease in the level of thyroid hormone, and stimulation of the anterior pituitary with a resultant hyperactive picture such as was observed in the present investigation. If, on the other hand, the picture is a result of continued inactivity, this would suggest that the primary cause of neoteny is hypophyseal, or perhaps that it must be sought for in the hypothalamus which regulates the hypophysis; in the former, certain changes in structure, hitherto not studied in detail, were observed.

The mechanism in delayed metamorphosis may also be explained in various ways, when the results are interpreted on the same principles as above. The high percentage of epithelium and the low percentage of colloid in the thyroid gland, together with the large amount of iodine, show that the uptake of iodine is not disturbed. Thus two possibilities remain: either the synthesis of thyroid hormone or its secretion is disturbed, or — and this seems more likely — tissue sensitivity to thyroid hormone is decreased. Furthermore, there is theoretically a third possibility, *viz.* the occurrence of

iodine deficiency; in this case the mechanism and the resulting picture would correspond to those of endemic goitre, and the high iodine value would be ascribable to an iodine deficiency in the thyroid. This alternative may be ruled out, however, since the control material fed with the same diet developed normally. In attempting to evaluate the remaining two alternatives we are again confronted by the dualistic aspect of the histological picture of the anterior pituitary. If this picture reflects a strong secretion of TSH, the first of the mechanisms outlined above is apparently involved: The secretion of thyroid hormone being disturbed, the protein-bound iodine in the tissues decreases, the anterior pituitary is stimulated, and consequently the thyroid gland hypertrophies and the iodine uptake increases. If, on the other hand, the anterior pituitary in this group is relatively inactive, as the results of previous investigators seem to indicate (12, 13, 5, 6, 8), it may be inferred that tissue sensitivity is decreased; consequently the activity of the thyroid gland increases in order to compensate the increased need for thyroid hormone, and the high hormone level inactivates the TSH system. In this case the hypertrophy of the thyroid gland would only be compensatory and independent of the hypophysis.

Summarizing, it must be admitted that the endocrine mechanism of neoteny and delayed metamorphosis cannot be explained on the basis of the results presented here. In both cases a disturbance of thyroid activity is undoubtedly involved, though not of the same nature. Investigations aimed at elucidating this question are in process, interest being mainly focussed on the histology of the hypothalamus and the iodine metabolism of the different types.

The possibility that there exist two different thyrotropins (3, 14) might offer an explanation to our results and explain the conflicting data found in the literature. This, however, needs further study.

#### SUMMARY

Two types of arrested metamorphosis in anurans have been investigated. In true irreversible neoteny, atrophy of the thyroid gland, a low iodine concentration in the colloid, and typical changes in the hypophysis (a marked increase in acidophilic granulation

and the disappearance of basophilic, PAS-positive substance) were observed. In cases where the experimentally induced delay in metamorphosis was reversible, the thyroid gland was hypertrophic, the iodine concentration of the colloid was high, and basophilic, PAS-positive granulation was encountered in abundance in the hypophysis.

The results show that the mechanism of arrested metamorphosis is not the same in the two groups, but they do not afford any conclusive explanation of the problem. Various possible interpretations are discussed.

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## OBSERVATIONS ON EXPERIMENTAL LATHYRISM IN THE RAT<sup>1</sup>

by

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Lathyrism in humans and livestock has been associated with the prolonged ingestion of large amounts of seeds of the *Lathyrus* genus (22). It is a form of spastic paraplegia with tremor, involving chiefly the legs. Human lathyrism occurs mainly in the Mediterranean region and especially in India (1, 2). The symptoms of experimental lathyrism in the rat are at first described by Geiger, Steenbock and Parsons (12). During the past years, experimental lathyrism in the rat has been subjected to intensive study (3, 4, 5, 6, 7, 8, 13, 14, 15, 18, 19, 20, 24). The main symptoms of experimental lathyrism in rat are kyphoscoliosis, paralysis, degenerative arthritis, dissecting aneurysm of the aorta, exostoses of the bones, incontinence of the urine and muscular atrophy.

The present study was undertaken in an effort to find out possible changes in some organs of rat, which have hitherto escaped any notices in the literature or of which controversial results have been presented. Special attention was given to the thyroid, thymus and adrenal glands.

### MATERIAL AND METHODS

Forty albino rats of the Wistar strain were used. Twenty were four-week-old male rats, weighing about 50 g. Ten of them received

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<sup>1</sup> Aided by a grant from The Sigrid Jusélius Foundation.

of experimental diet (A) and ten of control diet (B). Twenty rats were six-month-old females, weighing about 200 g. Ten of them received of experimental diet (A) and ten of control diet (B). The composition of the diets was as follows:

Diet A:

	Per cent
<i>Lathyrus odoratus</i> (Spencer) meal .....	50
Sucrose .....	24
Commercial casein .....	10
Brewery yeast.....	10
Mc Collum's salt mixture .....	4
Olive oil containing 0.21 mg of vitamin A, 0.26 IU of vitamin D, 10 mg of vitamin E, and 0.15 mg of vitamin K per kilogram of diet.....	2

Diet B: The same except that the sweet pea meal was substituted for commercial edible pea (*Pisum sativum*) meal.

Since, during the experiment, it was found that the diet B containing edible pea meal was not an adequate control diet in studying the thyroid activity, another series consisting of twenty rats was made. Ten male rats weighing about 50 g and ten female rats weighing about 200 g were fed with the ordinary diet of our laboratory (C), the composition of which was as follows:

Diet C:

	Per cent
Oat meal .....	24
Whole oat meal .....	24
Whole wheat meal .....	24
Rye meal .....	24
Brewery yeast .....	1.3
Calcium lactate .....	1.3
Cod liver oil.....	1
Table salt .....	0.4

From these rats, only the thyroid glands were studied. For each group food and tap water were available *ad libitum*. They were weighed weekly. One rat from each group was sacrificed by decapitation after a period of ten days. Thus, the last animals were killed 100 days after the onset of the experiment. An X-ray exami-



nation of the rats was performed at fiftieth day of the experiment. Immediately after sacrifice, the thyroid, thymus and adrenal glands were removed, weighed, embedded in in paraffin and stained with Mallory's azan. The percental proportion of the thyroid epithelium, colloid and stroma was determined using the histoquantitative method of Uotila and Kannas (23). The thoracic aorta was examined histologically using Weigert's elastin stain. Pieces from the skin of back, kidney, liver and stomach were taken, fixed in Bouin's fluid and stained with hematoxylin-eosin.

#### RESULTS

*Young Animals.* — The consumption of the control and experimental diets was roughly equal. The control animals thrive well with their regimen. They showed a weight gain of approximately 25 g during ten-days periods. At first, also the weight of experimental rats increased, but after reaching a weight of approximately 100 g, the gain of weight ended. At seventeenth day of the experiment one rat of the lathyrus-group died. Necropsy revealed ruptured thoracic aorta and hemothorax. This was the sole case of aortic rupture in our material.

After 30 days sweet pea diet severe skeletal lesions occurred.



Fig. 1. — Roentgenogram of a 90-day-old male rat fed on lathyrus diet for 50 days. Marked kyphoscoliosis and other skeletal changes.



Marked thoracic deformities, kyphoscoliosis, disc herniations and valgus of the forepaws were observed in all animals (Fig. 1). The animals were more or less paralytic. These changes became more pronounced towards the end of the experiment.

Sections taken from the kidney and stomach showed no microscopically discernible alterations. In the liver, small infiltrations of polymorphonuclear cells and small, apparently sterile necrotic areas were seen. These changes, however, were not present in all rats of the sweet pea group. The skin of the back was thinner and the amount of subcutaneous tissue reduced in the lathyrus-group as compared with those of the controls. In the wall of the aorta the elastic fibers seemed to have a normal appearance. In some cases there was a slight edema in the media of the thoracic aorta.

On microscopical examination of the adrenal glands of the lathyrus-group a hypertrophy of the cortical cells and numerous vacuoles in their cytoplasm were regularly found. The mean absolute weight of the adrenals was essentially the same in both groups, but because the rats of the control series were much larger, the mean relative weight of the adrenals in the sweet pea group ( $34.1 \pm 3.14$  mg/100 g) was significantly above that of the controls ( $19.6 \pm 2.29$  mg/100 g).

The thymus was clearly involuted already after ten days sweet pea diet, and this finding could be made in all the rats of the lathyrus-group. The mean absolute and relative weights of the thymus were as follows:

	Weight of the Thymus (mg)	Weight of the Thymus (mg/100 g of Weight)
Edible Pea Group . . . . .	$312 \pm 44$	$223 \pm 29$
Sweet Pea Group . . . . .	$82 \pm 19$	$91 \pm 27$

The differences were statistically significant (level 1—0.1 per cent).

The histological picture of the thyroid gland was substantially the same in the rats fed with sweet pea or edible pea. There was a redundancy of the epithelium and a paucity of the colloid indicating a thyroid active as far as the histological picture is concerned. In the control series for the thyroid gland fed on ordinary mixed food the histological picture of the thyroid was somewhat less

active. The mean absolute and relative weights of the thyroid and the percental proportion of the epithelium, colloid and stroma were as follows:

	Weight of the Thyroid (mg)	Weight of the Thyroid (mg/100 g)	Epithelium Per cent	Colloid Per cent	Stroma Per cent
Edible Pea Group	30.1 ± 8.8	20.7 ± 4.5	78.0 ± 1.9	11.6 ± 1.8	10.4 ± 0.8
Sweet Pea Group	17.2 ± 1.9	18.4 ± 1.2	78.7 ± 1.6	9.9 ± 1.5	11.4 ± 0.9
Mixed Food Group	18.1 ± 1.5	12.2 ± 1.0	74.2 ± 1.9	16.2 ± 1.2	9.6 ± 0.6

The differences between various groups were not significant statistically.

*Old Animals.* — Even in the groups of older rats the consumption of the control and experimental diet was approximately the same. There was a small and consistent weight gain in the control rats, whereas the weight of the animals of the lathyrus-group showed no substantial changes during the entire experimental period. There were no drastic skeletal changes, only small kyphoscoliosis could be observed in two rats of the sweet pea group. Histological examination of the liver, stomach, kidney, skin and wall of the aorta did not reveal any alterations.

The cortical cells of the adrenal gland in the rats of the lathyrus-group were slightly hypertrophied, but this hypertrophy was not as marked as in younger animals. There were no significant alterations in the mean absolute and relative weights of the adrenals in the lathyrus-group, although the mean relative weight in this group tended to be higher.

The thymus was involuted in the sweet pea group, but its atrophy was not as pronounced as in younger rats. The mean absolute and relative weights of the thymus were as follows:

	Weight of the Thymus (mg)	Weight of the Thymus (mg/100 g)
Edible Pea Group . . . . .	250 ± 25	115 ± 11
Sweet Pea Group . . . . .	169 ± 17	90 ± 9

The difference between the absolute weight of the thymus was statistically significant (level 1—0.1 per cent), but the difference between the relative weights was statistically not significant.

There were no differences in the histological picture of the thyroid between the sweet pea and edible pea groups. The amount of the epithelium was abundant, and respectively, the amount of colloid was small. The weights of the thyroids increased towards the end of the experiment in both groups. The thyroids of the rats which were killed from 80 to 100 days from the onset of the diets weighed over 100 mg. The histological picture of the thyroid of the rats fed on diet C was less active and the weights of the thyroids were much smaller. The mean absolute and relative weights of the thyroid and the percental proportion of the epithelium, colloid and stroma were as follows:

	Weight of the Thyroid (mg)	Weight of the Thyroid (mg/100 g)	Epithelium Per cent	Colloid Per cent	Stroma Per cent
Edible Pea Group	66.1 ± 13.7	29.0 ± 5.9	80.6 ± 1.5	7.4 ± 0.8	12.0 ± 1.0
Sweet Pea Group	65.4 ± 15.4	34.1 ± 7.4	81.6 ± 1.4	6.8 ± 0.5	11.6 ± 0.9
Mixed Food Group	21.0 ± 1.1	10.9 ± 0.8	75.8 ± 1.1	17.1 ± 1.0	7.1 ± 0.6

All the values of the mixed food-group differed significantly from those of the two other groups (level 1—0.1 per cent or 0.1 per cent).

#### DISCUSSION

Much work has been done for the isolation of the toxic material present in sweet pea seeds (9, 10, 11, 21, 25). The data recently presented strongly indicate that the toxic factor in sweet pea seeds is an aminonitrile.  $\beta$ -( $\gamma$ -L-glutamyl) aminopropionitrile (21) and  $\beta$ -aminonitrile (10) have been isolated from sweet peas, and it has been clearly demonstrated that these compounds, when fed to rats, produce skeletal changes similar to those caused by sweet peas (10, 25).

The skeletal changes observed in the present experiment are quite similar to those described by earlier investigators. Since the skeletal deformities were minimal or absent in older series, it seems to be probably that the toxic factor of sweet peas exerts its destroying action in growing bone and connective tissue. Ponseti and Shepard (20) assumed that these lesions are due to defective

formation or excessive destruction of the chondroitin sulphate of the ground substance.

The present observations on the alterations in the liver, if specific, are possibly explained by the detoxificative function of this organ.

Our observation regarding the hypertrophy of the adrenal cortex confirms the findings of Dasler (6). Also in his material the hypertrophy of the adrenals was not as marked in female rats as in males. It is in this connection interesting to note Stockman's (22) statement that the young human adult males are more susceptible to lathyrism than are women, elderly men or children.

In our material the feeding of sweet peas induced a pronounced involution of the thymus. Whether sweet peas have any direct thymolytic effect or whether the involution of the thymus together with adrenal hypertrophy can be explained as a stress effect, cannot be stated. Since the enlargement of the adrenals was relatively small, and the involution of the thymus very marked, the former assumption is perhaps more plausible.

Both sweet pea and edible pea diets caused an enlargement of the thyroid gland which was nearly directly correlated to the duration of feeding. Regarding sweet pea diet this observation can be explained by the fact, that, if the active toxic compound of sweet pea is an aminonitrile, the cyano group of this compound can be responsible for the goitrogenic activity of sweet peas. Cyanides are known to impose an obstruction to the completion of thyroid hormone thus causing an excessive production of thyrotropic hormone and thyroid hyperplasia.

That the goitrogenic factor of the edible pea diet must lie in edible pea seems to be probable. Van Middlesworth (17) has recently shown in rats, that the addition of casein, another component of our diet, to low iodine goiter producing diets prevented goiter and its withdrawal from these diets produced large goiters. The other components of this diet are in general used in rat diets, and it is very improbable that they could induce thyroid hyperplasia.

We have not found any notice in the literature on the goitrogenic properties of edible peas. Cabbage, Brussel sprouts, cauliflower, rape seed and soy beans has been shown to be goitrogenic under certain conditions (for references, see Means 1948). It seems to be

probable, that edible pea, like its related vegetable, sweet pea, contains a goitrogenic factor which can cause thyroid hyperplasia, at least when fed at a level of 50 per cent.

## SUMMARY

The effect of diet containing 50 per cent *Lathyrus odoratus* seeds on the organs of rat was investigated. Typical skeletal changes occurred. These alterations were more pronounced in growing rats than in adult rats. Necrotic areas in the liver were found. Adrenal cortex hypertrophied, in males more clearly than in females. The thymus showed a marked involution. Both control diet containing 50 per cent edible pea and sweet pea diet induced a hyperplasia of the thyroid.

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## EXERCISE STUDIES ON PARAPLEGICS AND BILATERAL ABOVE-KNEE AMPUTEES

by

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The disability of paraplegics and of bilateral above-knee amputees essentially restricts their possibilities to exercise. The large muscle groups of the legs are out of function. The muscles of the arms and of the shoulder girdle assume an essential rôle in the exercise still done. Moving about in a hand-propelled wheel-chair leads to a considerable hypertrophy of these muscle groups.

The hypertrophy of certain muscle groups does not, however, indicate anything about other aspects of physical fitness. Thus, the condition of the cardiovascular system is known to be largely independent of muscle strength. Therefore, one of the purposes of the present study was to compare the *pulse rate* of such disabled subjects in connection with standard ergometer work with that of normal controls.

Furthermore, it was asked, whether the disabled subjects did the ergometer work with a *mechanical efficiency* differing from that of the normal controls. Several studies have shown that training may improve the mechanical efficiency of muscular work (3, 12, 1). Moreover, the mechanical efficiency of pedalling with arms has been shown to be relatively low, ca. 18 per cent, while that of leg work varies between 20 and 30 per cent (9). A general experience is that training may be expected particularly to improve the mechanical efficiency of such kinds of work in which it primarily



is relatively low, whereas the mechanical efficiency may not be expected to improve essentially in activities commonly practised like walking (5). As the disabled subjects were accustomed to pedalling with their arms, they could thus have been expected to do the test with a mechanical efficiency superior to that of untrained controls.

The writers are not aware of any studies on the effect of muscle hypertrophy on its mechanical efficiency. This factor may also be of importance.

The mechanical efficiency of arm pedalling was therefore also included in the study.

#### MATERIAL AND METHODS

Experiments were made on twelve male paraplegics and on three amputees. The disability of the paraplegics was caused by transversal lesions of the spinal cord, obtained in war. The amputees had bilateral above-knee amputations, similarly because of having been wounded in war. As control subjects served 20 male students.

The upper arm circumference was measured at its thickest level, both the muscles relaxed and at the maximum voluntary contraction of the flexors. The thickness of the triceps skinfold was measured with a caliper at standard pressure. In this way the area of the skin and subcutaneous tissue could be subtracted from the transsectional area of the upper arm.

For determining the energy expenditure, the expired air was measured with the Kofranyi-Müller gasometer (8, 10). Oxygen and carbon dioxide were determined in the gas samples with the aid of Scholander micro gas analysis apparatus (11).

The resting metabolic rate and pulse rate were first determined with the subject sitting in the working position. Then the same determinations were repeated, while the subjects were pedalling a frictional ergometer (7) 5 minutes at each of two loads. With appr. half of the subjects the loads were 250 and 320 kg-m per min., with the rest 340 and 490 kg-m min. The energy expenditure was determined during the last minute of the work. The energy cost of the work was obtained by subtracting the resting metabolic rate from the total energy expenditure during work. The ratio of mechanical work and the energy cost of work, as a percentage, is the mechanical efficiency of work (6).



The recovery of the pulse rate (2, 4) was measured by counting the pulse from 15 seconds until 1 min. 15 sec. and subsequently until 2 min. 15 sec. after stopping work. The pulse was counted with the aid of auscultation.

## RESULTS

*Pulse Rate.* — The mean pulse rates at each load are shown in Table 1. The resting pulse rate of the disabled was relatively high,

TABLE 1

MEAN PULSE RATE AT REST AND AFTER EACH LOAD, DURING THE PERIODS 15"—1'15" (1) AND 1'15"—2'15" (2) AFTER STOPPING WORK. SIGNIFICANCE OF THE DIFFERENCES

	Disabled	Controls	Difference: Disabled — Controls
Rest	89.3	72.6	$16.7 \pm 3.7$ ( $t=4.47$ , $p<0.001$ )
250 kg/min. (1)	118.8	94.6	$24.1 \pm 7.3$ ( $t=3.29$ , $p<0.01$ )
(2)	106.0	85.1	$20.1 \pm 6.1$ ( $t=3.43$ , $p<0.01$ )
340 kg/min. (1)	140.9	108.6	$32.3 \pm 5.7$ ( $t=5.63$ , $p<0.001$ )
(2)	118.2	89.3	$28.9 \pm 5.9$ ( $t=4.90$ , $p<0.001$ )
490 kg/min. (1)	165.2	132.0	$33.2 \pm 9.3$ ( $t=3.59$ , $p<0.01$ )
(2)	132.7	103.4	$29.3 \pm 7.6$ ( $t=3.86$ , $p<0.01$ )

significantly higher than that of the controls. The same is true of the recovery rates, both during the first and during the second minute of counting. Evidently the pulse rate of the disabled rises at each load more than that of the controls, and the return of the pulse to the resting range seems to take more time. The difference between the disabled and the controls tends to increase with increasing load; graphically this means that the pulse rate of the disabled rises with a steeper slope than that of the controls, when plotted against the load. The difference between the 1st and 2nd minute counts tends to be higher in the disabled than in the controls. The pulse of the amputees did not differ from that of the paraplegics.

*Arm Muscles.* — After subtracting the area of the skin and subcutaneous tissue the transectional area of the upper arm bone and relaxed muscle of the disabled was on an average 66.4 sq.cm. and that of the controls 52.8 sq.cm. The difference of these values,

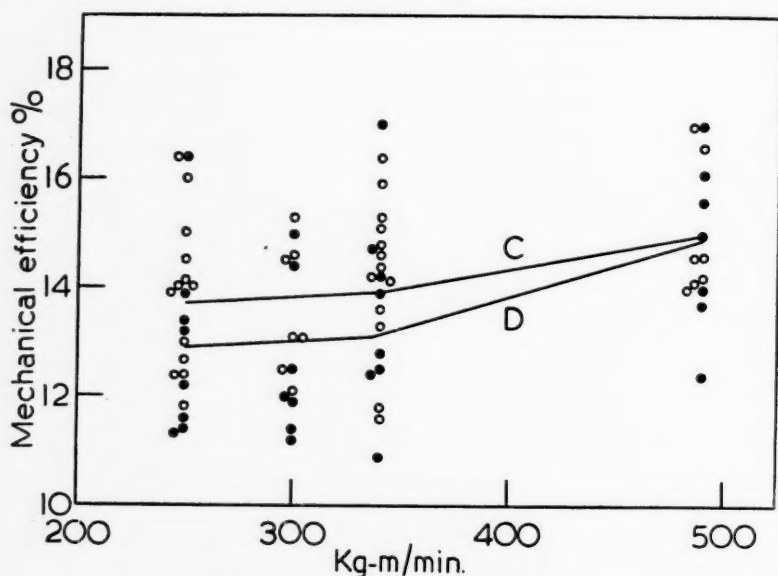


Fig. 1. — Mechanical efficiency of the disabled (●) and of the controls (○), with corresponding mean graphs (D=disabled; C=control).

$13.6 \pm 3.0$  sq.cm. was statistically highly significant ( $t=4.50$ ,  $p<0.001$ ). When the flexors were maximally contracted, the difference was larger still.

**Mechanical Efficiency.** — The mechanical efficiency of the pedalling varied between 12 and 17 per cent (Fig. 1). There was no marked difference between the disabled and the controls. The mean of the disabled was slightly lower at the lightest load used, but at the higher loads the mean values rose and came closer to each other. However, at none of the loads was the difference significant ( $p>0.05$ ). The scatter is so large that the tendencies observed must await further confirmation.

#### DISCUSSION AND CONCLUSIONS

The exercise *pulse* of the disabled was evidently considerably higher than that of the controls. Most probably they had a small stroke volume; in order to attain the cardiac output required they had to rely more upon raising their pulse rate. On the other hand, the relatively rapid initial fall — from the 1st to the 2nd

minute of counting — of the pulse after work may be ascribed to an adequate capillarisation of the working muscles and consequently to a relatively low concentration and rapid washing out of acid and other metabolites in the working muscles.

All the disabled subjects had exercised actively with the aid of handpropelled tricycles. The amount of exercise had probably become reduced some time before the experiments, as some of the subjects had started to use motordriven tricycles. To replace the loss of exercise, two had actively taken up table tennis. However, even with a keen effort, the amount of exercise these disabled get evidently is not sufficient to keep their cardiovascular fitness on the same level as that of healthy controls. The impossibility to use the large muscle groups of the legs seems to prevent an effective training of the heart. Whether the paraplegic condition by itself contributes e.g. to the high resting pulse, is not known.

The lack of sufficient exercise may also have contributed to the development of considerable overweight in most of the disabled subjects.

The mechanical efficiency is generally expected to improve with training. On the other hand, it might be expected that moving larger, hypertrophic muscles would decrease the mechanical efficiency of the muscles for external work, if there is any internal viscosity in the muscle. The present series does not speak in favour of any of these opposite hypotheses. Obviously, training and consequent hypertrophy had not essentially affected the mechanical efficiency of these muscle groups.

#### SUMMARY

The exercise tolerance and mechanical efficiency of arm pedalling was studied in 12 paraplegics, in 3 bilateral high leg amputees, and in 20 healthy control subjects. The disabled were accustomed to arm pedalling, whereas the controls were not.

The mean resting (sitting) pulse of the disabled was 89.3 per min. and significantly higher than that of the controls. At each load the pulse rate of the disabled during the periods 15"—1'15" and 1'15"—2'15" after stopping the work was also significantly higher than that of the controls. The difference tended to increase with increasing load. The fall of the pulse rate from the 1st to

the 2nd minute of counting was greater in the disabled, but their pulse rate during the 2nd minute was still farther above the resting level than that of the controls. The results were interpreted as indicating that the cardiovascular exercise tolerance of the disabled was poorer than that of the controls, in spite of the fact that training had caused their arm and shoulder muscles to hypertrophy to larger size than in the controls.

The mechanical efficiency of the disabled and of the controls did not differ significantly.

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## EFFECT OF THE DOSAGE OF SOME ANTIHISTAMINICS ON ADRENALINE RESPONSES OF SEMINAL VESICLE AND BLOOD PRESSURE<sup>1</sup>

by

M. K. PAASONEN,<sup>2</sup> O. J. KASSILA and N. T. KÄRKI

(Received for publication December 12, 1955)

The antihistaminic substances, in addition to their histamine antagonism, have many other properties of therapeutic importance. The ability in various tests to affect adrenaline responses is quite common to antihistaminics. Thus they can behave like adrenomimetic (especially chlorprophenpyridamine and mepyramine) or adrenolytic (phenindamine and promethazine) drugs and one drug can have both properties when used in a different kind of test, partly depending upon the dose (for ref. see 10, 2).

This work was mainly undertaken to show how closely the ability of antihistaminics to modify adrenaline responses is related to their dosage. This is demonstrated by using guinea pig seminal vesicle and cat blood pressure.

The following drugs were used:

Adrenaline hydrochloride, Orion Oy, Helsinki,

<sup>1</sup> We acknowledge with thanks the help of Lääke Oy., Manufacturing Chemists, Turku, for test animals, Schering Corp., Bloomfield, N. J., for chlorprophenpyridamine maleate («Chlor-trimeton»), Parke, Davis & Co. Hounslow, for diphenhydramine hydrochloride («Benadryl»), F. Hoffmann — La Roche & Co., Basle, for phenindamine hydrogen tartrate («Thephorin») and May & Baker Ltd., Dagenham, for mepyramine maleate («Anthisan») and promethazine hydrochloride («Phenergan»).

<sup>2</sup> M. K. Paasonen was at the time of this work a recipient of a grant from The Sigrid Juselius Foundation and takes this opportunity to express his gratitude.

Histamine acid phosphate, Burroughs Wellcome & Co., London, and Antihistaminics mentioned in the footnote of previous page.

Throughout the investigation doses of all substances were calculated as corresponding salt.

#### GUINEA PIG SEMINAL VESICLE

a) *In Vivo*. — In these tests we studied the effect of chlorprophenpyridamine and phenindamine on adrenaline responses in eight male animals weighing 450—750 g. Test animals were anesthetized by 1.5 g urethane subcut. or intraven. A cannula was tied into the jugular vein and all drugs were injected in this way in 0.2—0.3 ml of saline. Both seminal vesicles were exposed and such parts of visceral peritoneum which prevented the free movement of vesicles were removed, but care was taken not to disturb the circulation. Fixed on the operating table the animals were soaked in 38°C saline up to their neck. The threads from vesicles were directed by help of small pulleys to levers. The interval between the two successive adrenaline injections was five minutes.

The dose of antihistaminic was usually 2 mg/kg. This dose of chlorprophenpyridamine increased as a rule the adrenaline contraction (Fig. 1). The action was usually stronger when more antihistamine was administered. The response was usually increased by 50—100% (i.e., 50—100% higher dose was needed to elicit the pretreatment response). In these doses phenindamine prevented the adrenaline contraction (Fig. 2). This was true also in animals, which had received chlorprophenpyridamine before phenindamine. However, chlorprophenpyridamine did not increase the response of phenindamine-treated animals.

Cocaine hydrochloride (1 mg/kg) strengthened the reaction of seminal vesicle to adrenaline, but dihydroergotamine methanesulphonate (Sandoz) (0.5 mg/kg) blocked it effectively. It seemed to us, that the adrenaline reactions were weaker, when urethane was injected intraperitoneally instead of subcutaneously.

b) *In Vitro*. — The tests were made according to the Brügger's method (1) as modified by Järvinen (6). The seminal vesicles were perfused by aerated Locke solution in 25 ml baths at 37°C temperature. Adrenaline was added every 20 minutes, and left in the bath for one minute. When antihistamine was used, it was injected 19 minutes before adrenaline. The concentrations of antihistaminics were usually  $1 \times 10^{-9}$ ,  $1 \times 10^{-8}$ ,  $1 \times 10^{-7}$ ,  $1 \times 10^{-6}$  and  $1 \times 10^{-5}$ . Each antihistamine dose was tested once or more in each experiment.

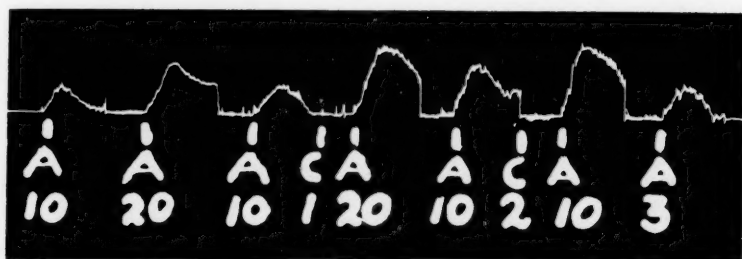


Fig. 1. — Guinea pig seminal vesicle *in vivo*. Adrenaline (A) in  $\mu\text{g/kg}$ . Chlorphenpyridamine maleate (C) in  $\text{mg/kg}$ .



Fig. 2. — Guinea pig seminal vesicle *in vivo*. Adrenaline (A) in  $\mu\text{g/kg}$ . Phenindamine hydrogen tartrate (P) in  $\text{mg/kg}$ .

**Chlorphenpyridamine.** — In concentration of  $1 \times 10^{-9}$  there was a slight increase in adrenaline contraction in about half of the cases. In  $1 \times 10^{-8}$  the increase was about 40%. In  $1 \times 10^{-7}$  it had no effect and in a concentration of  $2 \times 10^{-6}$  there was a clear adrenolytic effect (Fig. 3).

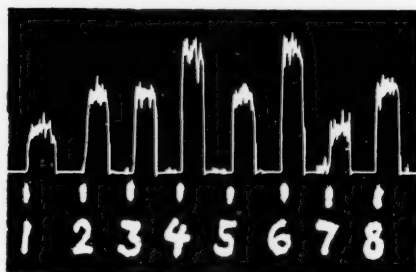


Fig. 3. — Guinea pig seminal vesicle *in vitro*. Adrenaline  $0.5 \times 10^{-6}$  at 1,  $1 \times 10^{-6}$  at 2, 3, 4, 5, 7 and 8,  $1.5 \times 10^{-6}$  at 6. Chlorphenpyridamine maleate  $1 \times 10^{-8}$  before 4 and  $2 \times 10^{-6}$  before 7.



*Diphenhydramine.* — In the concentrations of  $1 \times 10^{-9}$ — $1 \times 10^{-7}$  there was a small increase of adrenaline contraction. This effect was strongest with the concentration of  $1 \times 10^{-7}$  the average being 40%. The reaction in  $1 \times 10^{-6}$  was similar to that in  $1 \times 10^{-9}$ . On the other hand, in the concentration of  $1 \times 10^{-5}$  diphenhydramine had already a blocking effect of about 50%.

*Mepyramine.* — In dilutions from  $1 \times 10^{-9}$  to  $1 \times 10^{-7}$  there was only a small increase in adrenaline response. This was clearly demonstrated in  $1 \times 10^{-8}$  but even then it was only about 20%. In  $1 \times 10^{-6}$  there was an adrenolytic effect in some cases and this was noticeable in the concentration of  $1 \times 10^{-5}$  (50%).

*Phenindamine.* — At concentrations of  $1 \times 10^{-9}$ — $1 \times 10^{-8}$  it had no effect at all. In  $1 \times 10^{-7}$  there was about 70% blocking of the adrenaline contraction and in higher concentrations more than 100% higher adrenaline doses were necessary to elicit a response.

#### CAT BLOOD PRESSURE

Nine cats, 1.5—2.5 kg in weight, were anesthetized with intraperitoneally injected sodium amytal (60—80 mg/kg). All drugs were injected into the femoral vein through a cannula with 2 ml of warm saline. Blood pressure was measured from the left carotid artery with a mercury manometer. Chlorprophenpyridamine, phenindamine and in one test promethazine were given in increasing doses of 1  $\mu$ g, 10  $\mu$ g, 100  $\mu$ g, 1 mg and 10 mg many times or after 10 mg again 10 mg, then 20 mg and finally 25 mg. All these doses are calculated as mg/kg of body weight.

Chlorprophenpyridamine in doses of 1 and 10  $\mu$ g/kg did not affect adrenaline response. The reaction was slightly increased after injection of 100  $\mu$ g/kg and after this rather small dose especially the first part of the adrenaline reaction. The effect of larger doses was quite clear (Fig. 4). In no phase was the height or duration of response smaller than initial response or the reaction before the last antihistamine injection. Although after large doses of antihistamine the response was stronger (after the initial level of blood pressure, lowered by antihistamine, was reached) it began usually quite slowly and continued for a longer time. Generally, pressure after adrenaline injection begins to increase after some seconds, but here it began half a minute or more later. The lethal dose (the sum of all injections) of chlorprophenpyridamine was about 50 mg/kg for anesthetized cats.

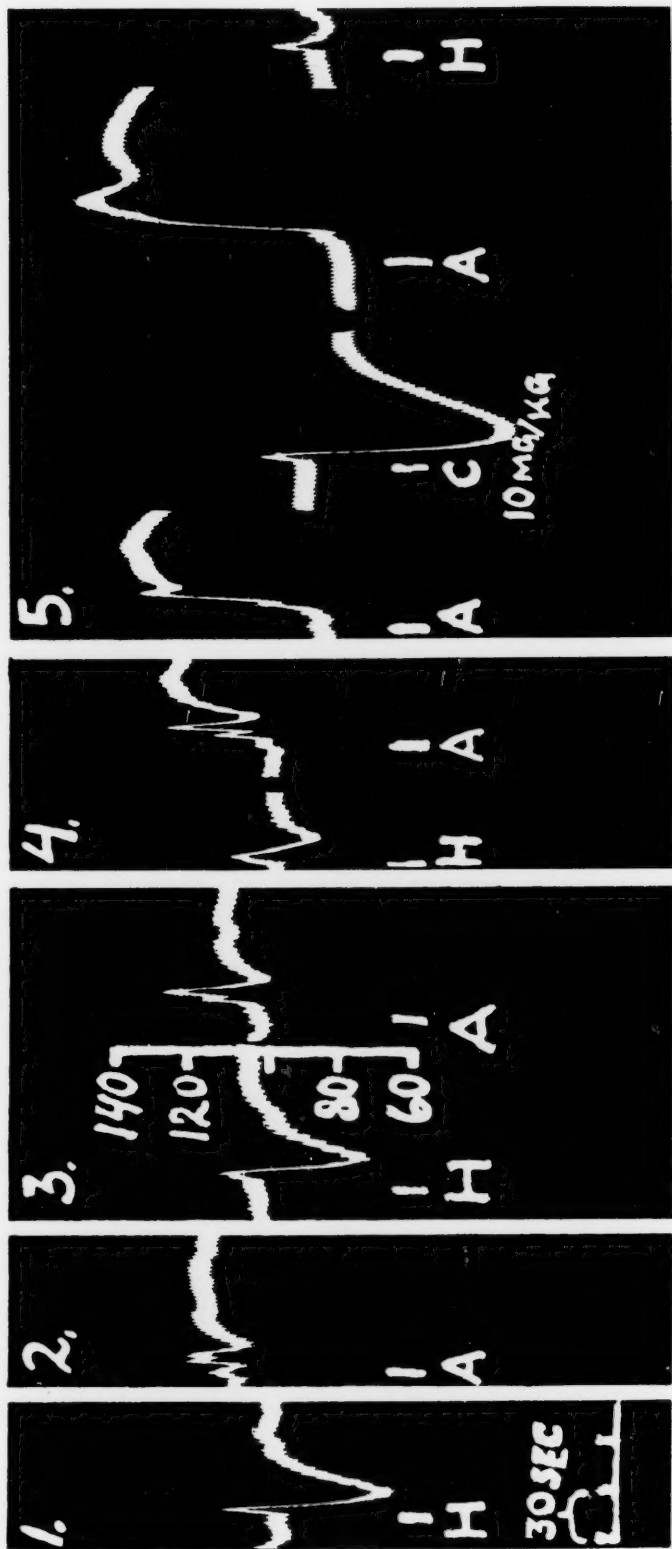


Fig. 4. — Cat, 1500 g. Blood pressure in sodium amytal anesthesia. Adrenaline (A) 2  $\mu\text{g/kg}$ , histamine (H) 0.5  $\mu\text{g/kg}$ . Between 2 and 3 chlorprophenpyridamine maleate (C) was administered 1, 10 and 100  $\mu\text{g/kg}$ . Between 4 and 5 C 10 mg/kg.

There was no potentiation of blood pressure responses to adrenaline by small phenindamine doses (1  $\mu\text{g/kg}$  and 10  $\mu\text{g/kg}$ ) or the action was a questionable sensitization. Usually the dose of 100  $\mu\text{g/kg}$  and always that of 1 mg/kg reduced adrenaline responses. A 50–100% higher dose was necessary to get the previous response after the latter phenindamine dose. The effect of phenindamine was increased by increasing the dose and after 10 mg/kg, 20 times more adrenaline had to be given in order to elicit the pretreatment reaction. There was no delayed starting of the response as after chlorprophenpyridamine administration. The lethal dose was from 40 to 50 mg/kg. There was no clear change in the blood pressure responses of adrenaline when a dose of promethazine, smaller than 1 mg/kg, was given. When 1 mg/kg or more was administered, the reaction was depressed.

Although the 1 mg/kg dose of antihistaminics mentioned was as a rule able practically to block histamine blood pressure reduction, adrenaline response was still possible to be changed by increasing the antihistamine dose.

#### DISCUSSION

It is known that at least some of adrenaline antagonists may have the opposite effect in small doses in some pharmacological tests (5, 12). The same is true for sympathomimetic amines which sensitize the effects of adrenaline in low concentrations but antagonise them in high ones (4,5). There are many examples of this kind of dual action and no doubt it is common also to antihistaminics. Reuse (11) has noted that mepyramine increases the adrenaline blood pressure reaction in a dose of 5–7 mg/kg in a chloralosed dog, but decreases it when 15 mg/kg is given.

*In vitro* tests on guinea pig seminal vesicle we demonstrated the same kind of behaviour with some antihistaminics. Phenindamine, however, which was previously noted to have adrenaline antagonistic effects in some tests (10) showed only antagonistic properties on seminal vesicle *in vitro* as well as in doses tried *in vivo*. The adrenomimetic nature of chlorprophenpyridamine noticed by us (10) and others (8), was stated on blood pressure tests and on seminal vesicle *in vivo*. Seminal vesicle *in vivo* seemed to be sensitized by chlorprophenpyridamine concentrations, which in

vitro were high enough to have adrenolytic effect. It is quite right, that all antihistaminics are adrenolytic when tried on isolated guinea pig seminal vesicle (2), but one have to remember, that at least some of them are adrenomimetic in low enough concentrations.

Our blood pressure tests do not support the assumption that reactive histaminemia modifies the adrenaline response. Concerning the »receptor» properties of antihistamines and pressor and depressor amines we refer to the interesting paper of Huidobro and Croxatto (3).

Antihistaminics and adrenaline are both agents used in the treatment of allergic conditions and it is necessary to be aware of the ability of the first mentioned drugs to modify the responses of the latter. In our previous work (9) the increase of the response of parenterally administered adrenaline due to phenindamine in human beings might have been the result of the adrenolytic action of this antihistamine, which may increase the absorption of adrenaline, by preventing vasoconstriction at the site of injection. Not only the action of injected adrenaline, but also the reaction of periferal organs to sympathetic stimulation may be changed by antihistamine substances (7).

When selecting an antihistamine to use on determination of adrenaline in order to prevent the effect of histamine possibly present in samples of biological origin, attention should be paid to the various effects of the different antihistamines on the adrenaline response.

#### SUMMARY

Chlorprophenpyridamine increased and phenindamine (about 2 mg/kg) decreased the adrenaline contractions of guinea pig seminal vesicle *in vivo*. By *in vitro* tests with these antihistamines and diphenhydramine and mepyramine, it could be shown that in weak concentrations (between  $1 \times 10^{-9}$  and  $1 \times 10^{-6}$ ) antihistamines, with the exception of phenindamine, strengthened the adrenaline response of guinea pig seminal vesicle although they all were adrenolytics in higher concentrations ( $1 \times 10^{-6}$  or higher). Chlorprophenpyridamine increased the cat blood pressure reaction to adrenaline, when the dose was 100  $\mu$ g/kg or more. Smaller doses

were ineffective. Phenindamine, in low doses, did not clearly change the adrenaline responses. In higher doses (often at 100  $\mu\text{g/kg}$  and always at 1 mg/kg) it had an adrenolytic action.

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## THE ANTIGENIC PROPERTIES OF THE BLASTOPORAL LIP OF THE TRITURUS GASTRULA<sup>1</sup>

by

TAPANI VAINIO

(Received for publication January 25, 1956)

In the last few years embryologists have to an increasing degree adopted immunological methods in the study of embryogenesis. With the aid of serological techniques, absorption, and *in vivo* tests, it has been possible to demonstrate the presence of specific organ antigens at an early stage of development, even prior to the morphological differentiation of the organs in question (1). In this research interest has mainly been focussed on the formation of new antigenic substances during the early phase of development, and on the relationship between the antigens of adult and embryonic tissues. Cooper (2), investigating the factors responsible for the cross-reactions of adult and embryonic tissues, was able to show that determinants of albumin and globulin type participate in these. Schechtman (3) observed that the antiserum against adult hen's proteins reacts with a saline extract of chick embryo. Furthermore he found by absorption that neither the albumin nor pseudoglobulin possesses any antigen that is not also present in the yolk; the first »non-yolk-like» antigens appear in the blood by the fifth day of incubation (4). Ebert (5, 6) detected organ-specific antigens at a very early stage of development of the chick embryo. Using the absorption technique, Clayton (7) studied the development of antigenic substances during gastrulation, neurulation, and the tail-bud stage of *Triturus alpestris*. In addition to common antigens,

<sup>1</sup> Aided a by grant from the »Emil Aaltonen Foundation».

he was also able to demonstrate specific mesodermal and ectodermal antigens. In extensive investigations concerning the temporal and spatial gradients of the immunochemical radicals, Ishikawa (8) showed that there is a gradual change both in the basic substances and in the surface radicals. He emphasized the significant role of the latter in morphogenesis, in particular substantiated by his *in vivo* tests.

In what follows, the question of antigenic epigenesis will not be discussed. The purpose of the present investigation was to reveal possibly existing differences in antigenicity between the blastoporal lip of the gastrula and the presumptive ectoderm at a stage when it may be assumed that the inductive agent is localized to the former. Many investigators have concluded that the mesoderm inductor is a protein (9). »Neuralizing» agent appears to be released from the cells as a result of unspecific processes also (10). The question of whether it is possible to demonstrate characteristic antigenicity in the inductor tissue, too, is interesting. Has the mesodermal antigenic factor detected by Clayton only morphological significance, or is it related to induction?

#### MATERIAL AND METHODS

The material of blastoporal lip and presumptive ectoderm used for immunization was obtained from gastrulae of *Triturus vulgaris*. The presumptive ectoderm and the invaginating part of the blastoporal lip were aseptically removed in Holtfreter-Ringer solution from IIInd stage (Harrison 10th and 11th stage) gastrulae (Fig. 1).

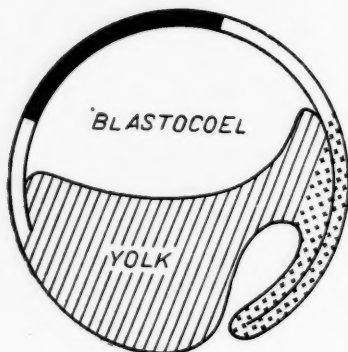


Fig. 1.



Lots consisting of blastoporal lip, or of ectoderm from 100 gastrulae were homogenized in a glass homogenizer in about 1 ml of Holtfreter-Ringer solution, and the suspension was centrifuged at 3000 r for about 20 minutes. The supernatant was used as such for immunization, and the residual after renewed suspension in a small volume of Holtfreter-Ringer solution. Four rabbits were immunized, two with ectoderm and two with blastoporal lip material. After administration of eight doses as described above, 15 ml of blood were drawn by puncture of the heart. The serum was used for serological tests. Three rabbits were used as controls.

The serological determinations were performed by precipitation. Antigen was prepared in the same way as the supernatant employed in immunization, except that physiological saline was used as diluent in a volume of 5 ml for 100 blastoporal lips or presumptive ectoderms, it having been shown by previous workers that the results are not affected if saline is used instead of Holtfreter-Ringer solution (7). Centrifugation was performed at 6000 r. for about half an hour. The lipid accumulating at the surface was removed with a sterile pad. The precipitation tests were in part performed as interfacial tests, in part by Oudin's technique (12) as modified by Muller (11). In the latter the procedure is as follows: Antiserum is mixed with the same amount of 4 per cent gum acacia, the mixture is alkalized to a pH of 7.2, and the antigen solution is stored on top of it in small glass tubes. In a heterogeneous material precipitation rings form in the gel, their number depending on the diffusion coefficient and concentration of the reactive antigen. The interfacial tests were performed in the usual way by storing antigen dilution on top of the antiserum. The results were read when the samples had been kept for 20 minutes at room temperature and for one day at  $+4^{\circ}\text{C}$ . The results of the diffusion tests performed by Muller's technique were read when the samples had been kept for one day, and for four days, at  $+4^{\circ}\text{C}$ . In the evaluation of the homogeneity of the antigen material the number of rings that had formed was used as index.

Absorption was performed in the same way as the interfacial tests. After standing overnight at  $+4^{\circ}\text{C}$  the reagents were mixed after 20 minutes at  $+20^{\circ}\text{C}$  and centrifuged at 3000 r for about half an hour. A more concentrated supernatant was used for absorption, *viz.* 100 gastrulae per ml of physiological saline.

## RESULTS

The results obtained by the precipitation technique are presented in Diagrams 1 and 2. All tests performed with control serum were negative, and so were also the tests performed with serum obtained from the rabbits prior to immunisation. The following abbreviations are used:

$P_s$  = the supernatant of the presumptive ectoderm

$P_r$  = the residual of the presumptive ectoderm

$O_s$  = the supernatant of the blastoporal lip

$O_r$  = the residual of the blastoporal lip

As appears from Diagrams 1 and 2, all rabbits except the one immunized against the residual of the presumptive ectoderm produced antibodies demonstrable by precipitation. It also appears from Diagram 1, that the homologous titre was relatively low, *viz.* 1/8, and about the same as the heterologous titre except that the titre of the anti- $O_s$  was higher with  $O_s$ -antigen than with  $P_s$ -antigen. Obviously the ectoderm and prospective mesoderm contain the same antigenic factors at the initial stage of gastrulation. On absorption with  $P_s$ -antigen the anti- $P_s$  titre dropped both with homologous and with heterologous material. By contrast, with the homologous material the anti- $O_s$  titre remained about the same, broadly speaking, showing only a very slight decline. On absorption with  $O_s$ -antigen the titres of both supernatant antisera employed displayed an equal drop. In the tests performed by Muller's technique (Diagram 2) the homologous titre was found to be higher in particular with the anti- $O_s$  serum. Of the residual antisera, the anti- $P_r$  yielded an uncertain reaction, whilst the anti- $O_r$  reacted with a definite titre both with the homologous and with the heterologous antigen material. It should be borne in mind, however, that anti- $P_r$  serum was obtained from one rabbit only. According to Oudin it is possible to evaluate the heterogeneity in antigen material by the number of rings observable in the gel column. In the present experiments it was found that the  $P_s$ - and the  $O_r$ -antigens yielded one (two) precipitation ring with the homologous and heterologous antisera, whilst the  $O_s$ -antigen definitely yielded two precipitation rings with the homologous antiserum, which in any case was one more than did the  $P_s$ -antigen with the homologous antiserum.

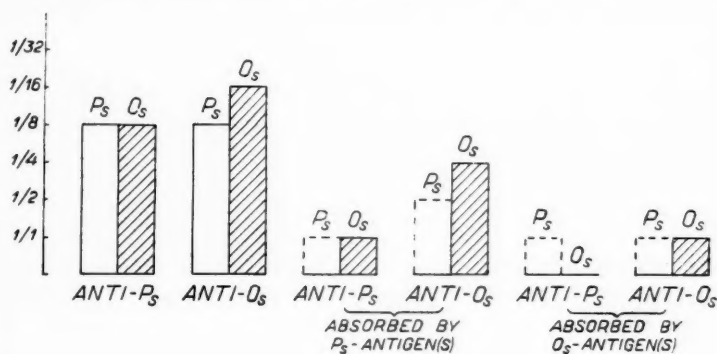


Diagram 1.

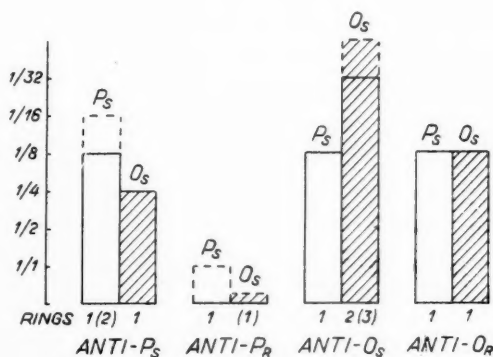


Diagram 2.

Furthermore it was found by Muller's technique that the anti- $Q_s$ , in contrast to the anti- $Q_r$ , anti- $P_s$  and anti- $P_r$  sera, reacted positively with antigen diluted in a guinea-pig bone marrow-saline solution, the titre being  $1/4$ . This interesting observation will be the object of continued investigations.

#### CONCLUSIONS

Owing to the limited number of experimental animals producing antibodies, it stands to reason that no far-reaching conclusions can be drawn from the present results. However, Clayton's observation that antigenic factors are present in the ectoderm and in the prospective mesoderm was confirmed. On absorption with heterologous

and homologous material a specific factor was demonstrable in the blastoporal lip, but not in the presumptive ectoderm. This finding was substantiated by the number of precipitation rings observed in tests by Muller's technique. On the basis of a study of this kind it is not possible to conclude whether the factor specific to the blastoporal lip bears any relation to the inductive agent(s). At the end of the operation period some *in vivo* tests were performed, pieces of the blastoporal lip kept in homologous, heterologous and control serum being implanted into the blastocoele. When the embryo had been incubated for two weeks, both mesodermal and neural inductions were definitely observed in all groups. Further investigations concerning the antigenic properties of the inductive agents will be carried out, principally using heterogeneous inducers, so that a more extensive material can be used.

In view of the fact that the antiserum against the soluble fraction of the blastoporal lip cross-reacted with the heterogeneous inductive material, it must be assumed that the bone marrow and the blastoporal lip of the gastrula contain the same antigenic factors. This result is tentative, however, and does not solve the question as to whether the cross-reaction here discussed is due to the presence of the same inductive substance(s) in both tissues, as might, perhaps, be assumed.

#### SUMMARY

The antigenic differences between the blastoporal lip and the presumptive ectoderm of the gastrula of *Triturus vulgaris* were investigated by immunizing rabbits against saline-soluble and insoluble fractions of blastoporal lip and presumptive ectoderm material. In precipitation tests antigenic differences between the blastoporal lip and the presumptive ectoderm were observed, which are referred to the presence of an antigenic factor specific to the former.

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## STUDIES OF AGGLUTINATION IN RHEUMATOID ARTHRITIS

### II

AN IMMUNOLOGICAL STUDY OF THE FACTOR IN RHEUMATOID SERA  
CAUSING AGGLUTINATION OF SENSITIZED ERYTHROCYTES

by

ODD WAGER<sup>1</sup>, PERTTI PALJAKKA and EVA ALAMERI

(Received for publication January 14, 1956)

In a considerable percentage of cases, the sera from patients with rheumatoid arthritis exhibit an extraordinary capacity to strongly agglutinate red cells sensitized with a subagglutinating dose of the homologous antibody. In recent years, this phenomenon has been subjected to numerous studies (1, 2, 4, 5—36).

The nature of the serum factor, responsible for this phenomenon, still is imperfectly understood. On ammonium sulphate fractionation it remains within the globulin fraction (18, 29), and in electrophoresis it moves with the  $\beta$ - $\gamma$ -globulins (18, 27). In a previous study (31) we fractionated rheumatoid sera by ethanol fractionation technique. A method of concentrating a protein fraction containing the factor, and consisting mainly of  $\beta$ -globulin, was presented.

On the basis of observations made in our previous studies, it was suggested that the factor may exert its action by enhancing the agglutination of the red cell antigen, and possibly also some other antigens by their homologous antibody (30). This factor was

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation

named «agglutination activating factor» (AAF), a designation originally employed by Waaler (29). The presence of the AAF in rheumatoid sera probably plays an important rôle in the agglutination of hemolytic streptococci by rheumatoid sera, exerting its activating action on the agglutination of the streptococcal antigen by its homologous antibody, present in small amounts in most human sera (30). Further corroboration to this theory on the mechanism of the streptococcal agglutination in rheumatoid arthritis has been given by the recent studies of Thulin (28). He was able to produce a reappearance of the agglutinating capacity on living streptococci of a rheumatoid serum, absorbed with streptococci, by combining to it another portion of the same absorbed serum, from which the AAF activity had been removed by bentonite treatment.

The AAF can be adsorbed to sensitized sheep cells and again released from them by elution at  $+56^{\circ}\text{C}$ . Non-sensitized sheep cells do not absorb the AAF (30).

An so far unsolved problem, frequently discussed by various authorities, is whether AAF is a property of normal human serum, which is enhanced in rheumatoid arthritis, or is it a pathological factor, also qualitatively different from normal human serum constituents.

In the present work, elucidation to this problem was sought by the use of immunological methods, known to possess an extraordinary degree of sensitivity and specificity.

Assuming that AAF, in accordance with its protein character, possesses antigenicity, rabbits were stimulated with different AAF preparations. The resulting immune sera were analysed for their capacity to neutralize the AAF activity of rheumatoid pooled serum. The effect of absorption with rheumatoid and normal human sera on the neutralizing capacity of the immune rabbit sera also was studied.

#### EXPERIMENTAL

Nine rabbits were originally subjected to stimulation with AAF active preparations, started in 1952. An attempt was made to produce specific anti-AAF immune sera. Therefore, AAF preparations, as completely as possible free from other human serum constituents, were employed for stimulation. These preparations were:



1) AAF active eluate, made from sensitized sheep cells, onto which AAF had been adsorbed from the globulin fraction of rheumatoid pooled serum. The elution procedure and the method of concentrating the AAF active globulin fraction, consisting mainly of  $\beta$ -globulin, were described in a previous paper (31).

2) Sensitized sheep cells, onto which AAF had been adsorbed from AAF active globulin fraction of rheumatoid pooled serum.

Stimulation with the above preparations alone and in combination with Freund's adjuvans (3), varying the route of injection and the schedule of stimulation, was continued from 1952 to the summer of 1954.

Bleedings taken at various intervals from the rabbits thus stimulated, did not show any measurable effect upon the AAF activity of a rheumatoid pooled serum. No precipitins for normal or rheumatoid human serum appeared either in the sera of these nine rabbits.

Five rabbits died during the stimulation period, some of them after a heart puncture, some without known reason. The remaining four rabbits (Nos. 494, 495, 503, and 506) were allowed to rest till the fall of 1954, whereupon the stimulation was resumed. This time, however, AAF active globulin fraction was employed for stimulation. The injection schedule during this new stimulation period was the following:

An AAF active globulin fraction, prepared in 1952 (31) and stored in lyophilized state in deep freeze since then, was diluted to its original volume to contain 0.5% of protein, as estimated by measuring the absorption at 280  $\mu$  with the Beckman spectrophotometer. This preparate consisted mainly of  $\beta$ -globulin (90%), of  $\gamma$ -globulin (6.6%), and of  $\alpha$ -globulin (3.4%). It caused agglutination of sensitized sheep cells in a dilution of 512, whereas no agglutination of non-sensitized sheep cells was caused by it.

One ml of this preparation was injected into the rabbits on four succeeding days (Oct. 19–22). The first injection was given by the intramuscular route, the others were given into the marginal vein of the ear. Ten days following the last injection about 50 ml of blood were collected from each of the rabbits by heart puncture. The sera were inactivated by incubating at  $+56^{\circ}\text{C}$  in a water-bath for 30 minutes, and then tested for the presence of precipitins against normal and rheumatoid human serum.

For this purpose, 0.2 ml of undiluted rabbit serum was layered with equal volumes of varying dilutions of the antigen serum. The immediate ring precipitation was recorded, then the reactants mixed with each other, and incubated for one hour at  $+37^{\circ}\text{C}$  in a water-bath, whereupon the final readings were performed.

The sera exhibited precipitin titers ranging from antigen dilution 1: 5000 to 1: 10000. No significant difference between the precipitin titers against normal or rheumatoid serum was observed. The sera of these four rabbits were then pooled and absorbed with non-sensitized sheep cells repeatedly, until all measurable agglutinins for non-sensitized sheep cells had been removed. This pooled rabbit immune serum was employed for the experiments to be described, and is hereafter called, for the sake of convenience, anti-R serum.

All the sera used in this study were inactivated by incubating at  $+56^{\circ}\text{C}$  in a water-bath for 30 minutes.

Estimation of AAF activity was performed following the method described elsewhere (30). In this method, sheep cells sensitized with  $\frac{1}{2} \times \text{MAD}$  (minimum agglutinating dose) of homologous rabbit anti-sheep cell serum are used. In the present work, all the sera studied were previously absorbed with non-sensitized sheep cells. Thus, the agglutination titer for sensitized sheep cells directly reflected the AAF activity of the serum to be tested.

*Effect of Anti-R Serum on AAF.* — Equal volumes of pooled rheumatoid serum (diluted 1: 2) and of various dilutions of anti-R serum were mixed, then kept at  $+37^{\circ}\text{C}$  in water-bath for an hour. No precipitate was formed, apparently due to the antigen excess in the mixture. The AAF activity of the mixtures were then tested. In simultaneous control experiments normal rabbit serum was substituted for the anti-R serum. All the sera had been previously absorbed with normal sheep cells, until all measurable normal agglutinins for sheep cells were removed.

From the data in table 1 it occurs that anti-R serum had a definite neutralizing effect on the AAF activity of the rheumatoid serum. This effect was proportionate to the amount of the anti-R serum present. Normal rabbit serum had no neutralizing effect on the AAF activity, observable under these conditions.

TABLE 1

AAF ACTIVITY OF POOLED RHÉUMATOID SERUM, TREATED WITH EQUAL VOLUMES OF VARIOUS DILUTIONS OF ANTI-R SERUM OR NORMAL RABBIT SERUM

Dilution of Anti-R Serum	Agglutination of Sensitized Sheep Cells										
	Final Dilution of the Rheumatoid Serum										4096
	4	8	16	32	64	128	256	512	1024	2048	
1: 2	2	2	1	1							
1: 10	2	2	2	1	1	1	—				
1: 50	3	3	3	2	2	2	1	—			
1: 250	3	3	3	3	3	2	1	1	1	—	
1: 1250	3	3	3	3	3	3	2	1	1	—	
1: 6250	3	3	3	3	3	2	2	1	1	1	—
1: 31250	3	3	3	3	2	2	2	2	1	—	
Dilution of Normal Rabbit Serum											
1: 2	3	3	3	3	3	2	2	1	—		
1: 10	3	3	3	3	2	1	1	—			
1: 50	3	3	3	3	2	2	2	1	1	—	
1: 250	3	3	3	3	3	2	1	1	—		
1: 1250	3	3	3	3	2	2	2	1	—		
1: 6250	3	3	3	3	2	2	2	1	1	—	
1: 31250	3	3	3	3	2	2	1	1	—		
Saline Control	3	3	3	3	3	2	1	1	—		

The rheumatoid serum, anti-R serum, and normal rabbit serum had all been absorbed with normal sheep cells, until no agglutination of normal sheep cells was caused by them in serum dilution 1: 2.

The degree of agglutination is designated in the table by figures from 1 to 3.

TABLE 2

AAF ACTIVITY OF POOLED RHEUMATOID SERUM, TREATED WITH EQUAL VOLUMES OF VARIOUS DILUTIONS OF ANTI-HUMAN  $\gamma$ -GLOBULIN SERUM (COOMBS' SERUM) OR ANTI-R SERUM

	Agglutination of Sensitized Sheep Cells							
	Final Dilution of the Rheumatoid Serum							
	8	16	32	64	128	256	512	1024 2048
Dilution of Coombs' Serum *								
1: 2	2	1	—					
1: 10	2	2	2	2	2	—		
1: 50	2	3	2	2	2	2	2	1 —
1: 250	3	3	3	3	2	2	1	—
Dilution of Anti-R Serum								
1: 2	2	2	1	1	—			
1: 10	2	2	2	1	1	—		
1: 50	3	3	2	2	2	1	—	
Dilution of Normal Rabbit Serum								
1: 2	3	3	3	3	2	2	1	—

\* The precipitin titer for human serum 1:10,000.

Thus, it was suggested by these results, that the neutralizing effect on AAF of the anti-R serum might be due to specific antibodies for AAF.

In order to investigate this question further, a control experiment was set up, in which an anti-human  $\gamma$ -globulin serum<sup>1</sup> (Coombs' serum) was substituted for the anti-R serum in the neutralization experiment like that described above. Data of this experiment are shown in table 2.

From the data in table 2 it can be seen that anti-human  $\gamma$ -globulin serum exhibited a neutralizing effect upon the AAF activity

<sup>1</sup> The  $\gamma$ -globulin preparation (Cohn fraction II—2, 3) was obtained from the State Serum Institute, Helsinki.

of the rheumatoid serum, fully comparable to that exhibited by the anti-R serum.

Next, fractional absorptions of the anti-R serum and the Coombs' serum with normal human and rheumatoid human sera were carried out. To 10 ml of undiluted rabbit serum to be absorbed, portions of 0.1 ml of the undiluted human serum were added. The tubes were incubated for one hour at  $+37^{\circ}\text{C}$  in a water-bath, then kept overnight in the ice box. The precipitate formed was removed by centrifugation, and the procedure repeated, until no precipitate was formed. Eight to ten fractional absorptions were required for this. The neutralizing effect of absorbed and non-absorbed portions of the anti-R serum and Coombs' serum upon the AAF activity of a pooled rheumatoid serum was then simultaneously tested.

Table 3 shows the results obtained in these absorption experiments.

From the data presented in table 3 it can be seen that absorption of the anti-R serum with normal or rheumatoid human serum had no measurable effect on the capacity of this serum to neutralize the AAF activity of a pooled rheumatoid serum. Absorption of the Coombs' serum with rheumatoid serum also remained without any effect on the neutralizing capacity of this serum.

#### DISCUSSION

In this study, a definite neutralizing effect upon the AAF was shown to be present in a pooled serum from rabbits, stimulated with the globulin fraction of a pooled rheumatoid serum. In this fraction, consisting mainly of  $\beta$ -globulin, was contained the bulk of the AAF activity of the rheumatoid serum. However, a fully comparable neutralizing power on the AAF was exerted by the serum of rabbit, stimulated with the  $\gamma$ -globulin fraction of a pooled normal human serum (Coombs' serum). Normal rabbit serum, under the conditions studied, did not possess any measurable effect upon AAF.

It must be born in mind, however, that both globulin preparations, in addition to their main component, also contained other globulin components with antigenic activity.

TABLE 3

EFFECT OF FRACTIONAL ABSORPTION WITH NORMAL OR RHEUMATOID HUMAN SERUM OF THE ANTI-R SERUM AND THE COOMBS' SERUM UPON THE NEUTRALIZING CAPACITY OF THESE SERA ON THE AAF ACTIVITY OF A POOLED RHEUMATOID SERUM

		Agglutination of Sensitized Sheep Cells									
		Final Dilution of the Rheumatoid Serum									
		8	16	32	64	128	256	512	1024	2048	4096
Dilution of Anti-R Serum											
Anti-R Serum, Non-Absorbed	1: 2	2	—								
	1: 10	1	—								
	1: 50	2	2	2	2	2	2	1	1	—	
Anti-R Serum, Absorbed with Normal Human Serum	1: 2	1	—								
	1: 10	2	1	—							
	1: 50	2	2	2	2	2	2	2	2	1	—
Anti-R Serum, Absorbed with Rheumatoid Human serum	1: 2	—									
	1: 10	1	1	1	—						
	1: 50	2	2	2	2	2	2	2	1	—	
Dilution of Coombs' Serum											
Coombs' Serum, Non-Absorbed	1: 2	—									
	1: 10	—									
	1: 50	2	2	2	2	2	2	2	—		
Coombs' Serum, Absorbed with Rheumatoid Serum	1: 2	1	—								
	1: 10	1	—								
	1: 50	2	2	2	2	2	2	2	—		
Saline Control		3	3	3	3	2	2	2	2	2	1 —

On the basis of observations made in our previous studies (30), it was suggested that AAF might be a property of normal human serum, which is enhanced in rheumatoid arthritis. The behaviour of the AAF in the neutralization experiments here described is in accordance with this view. By immunological means, we were not able to demonstrate any qualitative difference of AAF, as compared to normal human serum constituents. On the other hand, the results obtained suggest that the neutralizing power of the rabbit immune sera studied was caused by antibodies, formed by stimulation of the rabbits with antigen or antigens contained in the globulin fraction of human serum. Whether these antibodies were directed specifically against AAF or against some other antigen, necessary for the action of AAF, cannot be concluded on the basis of this study. Absorption of the rabbit immune sera with normal or rheumatoid serum, until all visible precipitins had been removed, did not result in any measurable decrease in the neutralizing power of these sera on AAF. Thus, the antibodies causing neutralization of AAF apparently are not identical with anti-human precipitins.

It is interesting to note that the AAF activity is liable to the effect of antigen antibody reactions. This circumstance urges a further use of immunological methods in the study of AAF. The availability of AAF preparations, purer and more potent than those so far achieved, would offer a great advance for these studies.

#### SUMMARY

The factor (AAF) in the sera of patients with rheumatoid arthritis, causing agglutination of sensitized erythrocytes, was investigated by immunological methods.

Serum from rabbits, stimulated with globulin fraction of rheumatoid or normal human serum, exhibited a definite neutralizing effect upon the AAF, whereas normal rabbit serum did not so.

The neutralizing power of the rabbit immune sera was not caused by the anti-human precipitins contained in these sera.

The results were discussed.

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## EFFECT OF POLYPHLOROGLUCINOLPHOSPHATE ON INFLUENZA VIRUS AND ON CHICKEN RED CELLS<sup>1</sup>

by

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Chemotherapeutic attempts to control viral infections have been concentrated mainly on the use of substances which interfere with the action of some enzymes (2). With this in mind an attempt was made to limit influenza virus infection with polyphloroglucinolphosphate (PPP), introduced as an alkaline phosphatase and hyaluronidase inhibitor (1, 3). The present paper describes some preliminary results of the experiments which show that polyphloroglucinolphosphate interferes in influenza virus infections in embryonated eggs. Since the substance in question, however, also affects viruses *in vitro* it is difficult to decide at this stage whether polyphloroglucinolphosphate interferes with the actual infectious process caused by the virus in or on the cells. Some observations on the «hemagglutinating» capacity of polyphloroglucinolphosphate are also presented.

*Methods.* — Influenza A strain A/Finland/1/51 was used in all the experiments. The allantoic fluid used in infecting the eggs and in the hemagglutination tests was obtained from eggs infected with a  $10^{-4}$  dilution of allantoic fluid. The age of the embryos at the time of inoculation was 10–12 days, the incubation temperature  $+37^{\circ}\text{C}$ . The dilution fluid was bacteriological broth.

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

The hemagglutination tests were carried out on plastic plates, as described earlier (5), and 0.5 per cent chicken cells were used. Cholera vibrio filtrate, manufactured by N. V. Philips-Roxane, Amsterdam, was used according to instructions of the factory.

Polyphloroglucinolphosphate. In the experiments, both the commercial preparation «Dealyd, Leo» and a concentrated preparation supplied by courtesy of the medical factory Leo (Helsingborg, Sweden) were used. The former contains 4 per cent PPP with phenylmercuric-nitrate 1/100,000. The concentrated preparation (20.6 per cent) contained no preservatives. The commercial preparation was dialysed for one week before use, with frequent changes of Hanks's solution. Under prolonged dialysis the preparation loses more than half of its brownish-red color. No essential differences were observed between the action of dialysed and undialysed preparations, although the former seemed to have a somewhat weaker action. If not otherwise stated, in all experiments PPP was diluted in bacteriological broth.

#### RESULTS

In the first experiments with polyphloroglucinolphosphate in embryonated eggs a striking inhibition of hemagglutinin production was observed. When, however the preparation was found to interfere with hemagglutination the effect of the preparation on the chicken red cells was studied.

*Effect of Polyphloroglucinolphosphate on Chicken Red Cells.* — When the preparation was titrated as an hemagglutinating agent on plastic plates «hemagglutination» was observed which could not be easily differentiated from virus agglutination. In the titration series, however, a «negative» zone appeared in the highest concentrations of the preparation. At the concentration level of about 1mg/ml a «negative button» is observed, after which agglutination can be seen up to the concentration level of about 0.01 mg/ml. This is the case when the diluent is buffered saline. When, however, the titration was carried out in the presence of chicken serum treated with 1 per cent cholera vibrio filtrate, the hemagglutination produced by the preparation was inhibited. Because the virus agglutination was not affected by the chicken serum treated with cholera vibrio filtrate, the agglutinations due to the preparation and in-

fluenza virus could be separated. In the following *in vitro* experiments the effect of PPP on red cells has been eliminated by chicken serum treated with cholera vibrio filtrate.

Taking into consideration the reliability of the hemagglutination tests in egg experiments, 0.5 ml of 1 per cent PPP was injected into allantoic sacs of several eggs in repeated experiments. After 24 hours' incubation of the eggs at +37°C the hemagglutinating capacity of the allantoic fluids was tested without the addition of chicken serum. In no one egg were hemagglutinins found, and the resuspended cells of negative »buttons» were agglutinated by four agglutinating units of the influenza virus. Thus PPP at the highest concentration used in the following *in vivo* experiments does not affect hemagglutination tests in egg experiments.

#### IN VITRO EXPERIMENTS

Table 1 shows the *in vitro* inactivation of the influenza virus by two concentrations of PPP.

TABLE 1

THE INACTIVATION IN VITRO OF HEMAGGLUTINATING CAPACITY AND OF INFECTIVITY OF INFLUENZA VIRUS IN THE PRESENCE OF POLYPHLOROGLUCINOLPHOSPHATE. INCUBATION TIME AT 37°C 24 HOURS. THE TITERS ARE EXPRESSED AS LOGARITHMS OF RECIPROCAL OF DILUTIONS. ID<sub>50</sub> % IS CALCULATED ACCORDING TO REED AND MUENCH. PPP WAS DILUTED IN NORMAL ALLANTOIC FLUID BEFORE MIXING WITH THE SAME VOLUME OF VIRUS ALLANTOIC FLUID.

	Titer of Incubated Control	Titer after Incub. with 0.05 % PPP	Titer after Incub. with 0.005 % PPP
Hemagglutination ..	3.0	0.9	1.2
Infectivity (ID <sub>50</sub> %)..	8.0*	5.2*	6.5*

\* six eggs were used per dilution.

Table 1 shows that after an incubation of 24 hours with 0.05 per cent polyphloroglucinolphosphate the hemagglutinating titer of the allantoic fluid decreased 2.1 log. units and the infectivity 2.8 log. units. With 0.005 per cent of the preparation the corresponding decreases were 1.8 and 1.5 log. units.

The results suggest that the inactivation rates of both hemagglutinating capacity and infectivity are of the same order. In other experiments this could be confirmed.

## IN VIVO EXPERIMENTS

*Toxicity of the preparation.* — When injected as a 4 per cent solution into allantoic and yolk sacs, 0.4 ml did not bring about any higher mortality than was observed in the controls. Normal chickens were hatched from the eggs. Bigger doses were not tried. This suggests that the toxicity of the phloroglucinolphosphate is not high in embryonated eggs. In the following experiments the highest dose injected into the eggs was 0.5 ml of 1 per cent solution, less than  $\frac{1}{3}$  of the dose in the toxicity tests.

*Effect of Polyphloroglucinolphosphate in Depressing the Infectivity and Hemagglutinating Titer of Allantoic Fluids of Embryonated Eggs Infected with Influenza Virus.* — Table 2 shows the *in vivo* depression of infectivity and hemagglutinating titer of allantoic fluid when PPP was injected into the allantoic sac before the virus.

TABLE 2

THE DECREASING EFFECT OF POLYPHLOROGLUCINOLPHOSPHATE ON THE HEMAGGLUTINATING AND INFECTIVITY TITER OF EGGS INFECTED WITH INFLUENZA VIRUS. INCUBATION TIME 24 HOURS. THE PREPARATION WAS INJECTED FIVE MINUTES BEFORE THE VIRUS. THE TITERS ARE EXPRESSED AS LOGARITHMS OF RECIPROCAL OF DILUTIONS. ID<sub>50</sub> % IS CALCULATED ACCORDING TO REED AND MUENCH. THE DOSE OF VIRUS IN THE INOCULUM WAS 300 ID<sub>50</sub> %.

	Titer of Pool of 9 Control Eggs	Titer of Pool of 8 Eggs Infected after 0.5 ml of 1 % PPP	Titer of Pool of 9 Eggs Infected after 0.5 ml of 0.1 % PPP
Hemagglutination	3.3	0.3 no egg showed hemagglutinins	0.3, no egg showed hemagglutinins
Infectivity (ID 50 %)	9.5*	5.6*	6.7*

\* six eggs were used per dilution.

Table 2 reveals a very marked effect of PPP. The hemagglutination titer is completely lost after injecting both 0.5 ml of 1.0% and 0.1% of the preparation into the allantoic sac before the virus. The depression of the hemagglutination titer as compared with the control is accordingly of the order of three or more log. units. The decreases in the infectivity titer due to the presence of the preparation are 3.9 and 2.8 log. units correspondingly.

Assuming that the preparation is diluted about 10—20-fold after injection into the allantoic sac (0.5 ml injected into about 5—10 ml

of allantoic fluid) and that the preparation remains and is not destroyed in the allantoic sac, the working dilutions in the allantoic sac are of the order of 0.1—0.05 and 0.01—0.005 per cent or at the same level as in the experiment presented in Table 1.

In repeated tests it was observed that the inhibition of hemagglutinin production was dependent on the dose of preparation, the timing of the injection of PPP and virus, and on the incubation time after the inoculation of virus.

TABLE 3  
THE EFFECT OF POLYPHLOROGLUCINOLPHOSPHATE ON THE PRODUCTION OF HEMAGGLUTININS. INCUBATION TIME 48 HOURS.

Dose of PPP	Time of Injection of PPP	ID50 % of virus in Inoculum	Hemagglutinins in Eggs
0.5 ml 1 %	—5 min.	10 <sup>2</sup>	— — — — — + + D D
" "	" "	10 <sup>5</sup>	— + + + + + + D D
" "	+240 min.	10 <sup>2</sup>	— — + + + + + D
" "	" "	10 <sup>5</sup>	+ + + + + + + D D
—	—	10 <sup>5</sup>	+ + + + + + + D D

+ = hemagglutinins    — = no hemagglutinins    D = died

When incubation time of 24 hours after the injection of virus was used, no hemagglutinins were found in eggs in spite of 10<sup>4</sup> ID50% of virus in inoculum. The dose was 0.5 ml of 0.1 per cent PPP diluted in bacteriological broth. Controls showed hemagglutinins of full titer. Some of the experiments with 48 hours incubation time are set up in the following tables.

Table 1 shows that hemagglutinin production in the experimental conditions presented was only prevented in a part of eggs.

TABLE 4  
THE EFFECT OF POLYPHLOROGLUCINOLPHOSPHATE ON THE PRODUCTION OF HEMAGGLUTININS. RESULTS WITH DECREASING DOSES OF THE PREPARATION. INCUBATION TIME 48 HOURS. 100 ID50 % OF VIRUS.

Dose of PPP	Time of Injection of PPP	Hemagglutinins in Eggs	Hemaggl. Titer of Positive Fluids
0.5 ml 0.5 %	—5 min.	— — — — — + + + +	1/32
" 0.125 %	"	— — — + + + + + D	1/256
" 0.03 %	"	— + + + + + + D D D	1/2048
—	—	+ + + + + + + D D	1/2048

It must be noted that the influenza virus strain in question regularly killed 1 or 2 eggs out of ten when incubation was continued for 48 hours.

According to Table 2, injection of 0.5 ml of 0.125 per cent polyphloroglucinolphosphate into the allantoic sac has a clear effect on hemagglutinin production when the incubation time of 48 hours is used.

#### DISCUSSION

The results of the experiments with polyphloroglucinolphosphate are not sufficient to provide an explanation of the depressing effect of the preparation on the hemagglutination and infectivity titer. In some experimental conditions this can be 3—4 log. units in embryonated eggs. The *in vitro* tests suggest that at least a part of the effect is due to the direct action of the preparation on the virus in the allantoic sac. The similarity of the inactivation rate *in vitro* of both hemagglutinins and infectivity seems to suggest that PPP forms combinations with virus particles and decreases the number of free virus units. It is interesting that PPP retains its activity *in vivo*. The much greater effect of the preparation with only 24 hours' incubation suggests, however, that some activity of the preparation is lost during prolonged incubation after infection with virus. The possibility that PPP also has some action on the entry of the viruses into or their release from the cells cannot be denied. Some antiviral activity of naphthol and phenol derivatives, which have a hyaluronidase inhibiting effect, has been recently observed (4). It is interesting that also other polymerized preparations such as sulphonic acid substituted polyureas (5), polymerized sodium salts of substituted benzoic sulphonic acids (9), and other polysulphonic acid derivatives (6, 7) have an antiviral effect.

#### SUMMARY

A report is made of experiments on the effect of polyphloroglucinolphosphate on influenza virus infection in embryonated eggs. The preparation has been introduced as a hyaluronidase and alkaline phosphatase inhibitor. In some experimental conditions a depression of the infectivity and hemagglutination titer of 3—4



log. units was observed when eggs were treated with the preparation before injecting the virus. On the other hand, polyphloroglucinolphosphate also has an effect on the influenza virus *in vitro*, and it is possible that the effect *in vivo* is of the same pattern.

Polyphloroglucinolphosphate has also an hemagglutinating effect on chicken red cells.

The mechanism of the effect of the preparation is discussed.

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## BIOLOGICAL ACTIVITY OF L-TRI-IODOTHYRONINE AND L-THYROXINE

OBSERVED AS RATE ACCELERATION OF THE ISOLATED RAT AURICLE

by

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Tri-iodothyronine, a compound closely related chemically to thyroxine, was recently discovered by Gross and Pitt-Rivers (7, 9). Various experiments have shown it to be biologically more active than thyroxine (2, 3, 6, 8, 10, 13, 17). In another paper we have reported a persistent accelerated rate in the isolated spontaneously beating rat auricle after treatment of the test animal with l-tri-iodothyronine (12). The above-mentioned test series was only qualitative and did not indicate any quantitatively marked difference in the activity of tri-iodothyronine and thyroxine. The purpose of the present study is to show if it is possible to detect any differences in the biological activity of l-tri-iodothyronine and l-thyroxine on the basis of rate acceleration in isolated rat auricles. To this end, the rats were treated with various doses of the respective substances before the auricle was isolated and examined.

### METHODS AND RESULTS

Seventy female albino rats were used as the test animals. Their average weight was 161 g. The weight of all but six rats was within the range 145 to 175 g. The auricular preparation was isolated and its rate of spontaneous beats at various temperatures was recorded isometrically as published previously (11, 12). The compounds administered to the rats on three successive days before the experi-

TABLE 1

CYCLE LENGTH (msec) AFTER ADMINISTRATION OF L-TRI-IODOTHYRONINE

Temperature °C	Control	Tri-iodothyronine Dose (mg daily)					
		0.01	0.03	0.05	0.10	0.40	1.00
25	570	415	362	352	346	313	354
27	428	348	274	295	279	264	276
29	330	288	244	240	233	219	238
31	274	253	203	205	204	193	201
33	222	201	176	173	179	166	167
35	189	183	154	150	156	146	146
37	162	155	134	125	136	125	127
39	141	135	120	112	118	112	114
41	122	115	105	100	105	101	102

ment were l-tri-iodothyronine (Glaxo) and l-thyroxine (British Drug). Groups of five to seven animals received equal doses of the respective substance.

The results of the l-tri-iodothyronine tests are seen in Table 1 and Figure 1. Table 1 is compiled from the values of cycle length. The figures represent mean values obtained from the preparations of the various groups. Various doses of l-tri-iodothyronine were administered. The greater the dose the shorter was the cycle or the higher the rate of beat up to a certain limit. This limit was reached with a daily dose of 0.03 or 0.05 mg of tri-iodothyronine. Larger doses did not result in additional shortening of the cycle. In Figure 1

TABLE 2

CYCLE LENGTH (msec) AFTER ADMINISTRATION OF L-THYROXINE

Temperature °C	Control	Thyroxine Dose (mg daily)				
		0.10	0.25	0.40	1.00	1.60
25	570	428	380	350	397	348
27	428	357	335	293	319	285
29	330	296	278	245	259	235
31	274	252	238	211	220	200
33	222	210	192	173	178	173
35	189	177	171	149	152	152
37	162	155	142	131	131	133
39	141	135	122	117	118	116
41	122	122	106	102	102	101

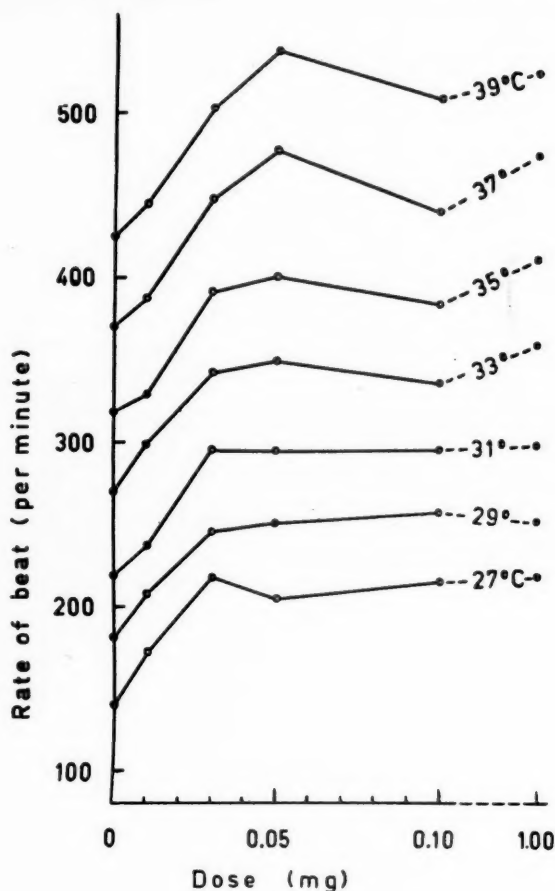


Fig. 1. — Rate of beat after l-tri-iodothyronine treatment. The dose given was injected on three successive days.

a part of the results is presented in the form of rate curves. The rate curves are similar at every temperature studied.

The effect on cycle length and rate of various doses of l-thyroxine can be seen in Table 2 and Figure 2. The changes were qualitatively similar to those after l-tri-iodothyronine administration. The maximum effect was obtained when the dose of l-thyroxine was 0.4 mg daily.

From a comparison of the results of the l-tri-iodothyronine and l-thyroxine series it appears that the maximum rate was equally

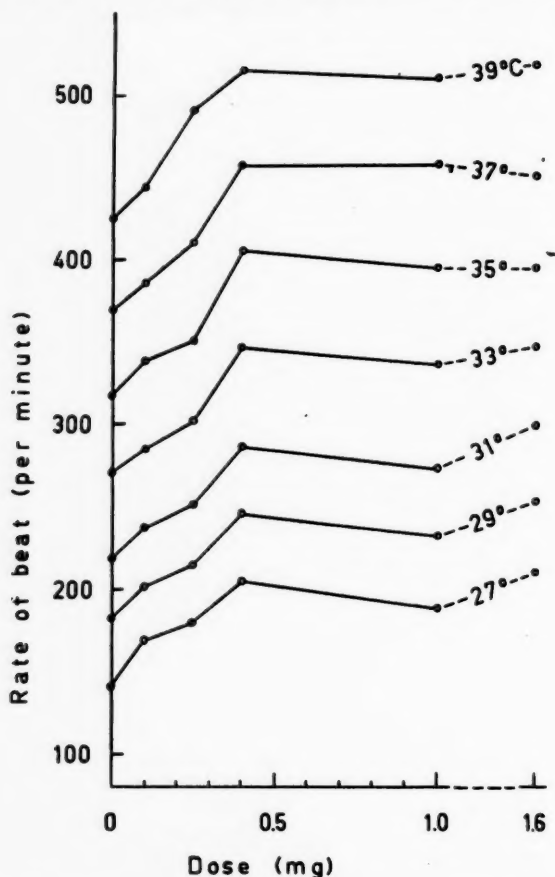


Fig. 2. — Rate of beat after l-thyroxine treatment. The dose given was administered on three successive days.

high with both substances. The maximum action of l-tri-iodothyronine was reached with a dose about one tenth that of l-thyroxine. On the weight basis, l-tri-iodothyronine appeared to be ten times as active as l-thyroxine in the test conditions of the present study.

#### DISCUSSION

The relationship of the rate of beat to the dose of the compound administered was in agreement with a common dose-effect curve (e.g. 14). After maximum effect, increased dosage failed to change

the result any further. This observation verifies the assumptions concerning the effect of thyroxine and related substances which were put forward in previous studies (12, 18) where the rate of beat was independent of the thyroxine dosage. All the experiments of the present study were made after injection of the substances on three successive days, this being the procedure that was expected to give the greatest effect (4, 5). It was found that l-tri-iodothyronine was 10 times as active as l-thyroxine. From goitre-prevention test with rats Gross and Pitt-Rivers (10) found that l-tri-iodothyronine possessed 5 times the activity of l-thyroxine. According to Tomich and Woollet (17) this ratio was 7.4 : 1 in rats. Furthermore they found by measuring the oxygen consumption in rats an activity ratio 5.1—5.3 : 1, and in mice a basal metabolic rate ratio 4.5 : 1 in favor of l-tri-iodothyronine. In the treatment of human myxoedema l-tri-iodothyronine has been found to be four to five times as effective as l-thyroxine (13). As inhibitors of pituitary thyrotrophic hormone secretion, using repeated daily doses, a ratio of 1.29 : 1 (weight for weight) has been found for the relative activity tri-iodothyronine and thyroxine (3). The galactopoietic action of tri-iodothyronine in cows has been found to be only slightly more effective than that of thyroxine (1). In the regulation of functions influenced by the thyroid it seems possible that there are certain quantitative differences in the effects of tri-iodothyronine and thyroxine. Apparently sino-auricular rhythm is one of the phenomena most sensitive to tri-iodothyronine. There are observations which clearly suggest that tri-iodothyronine is the peripheral thyroid hormone and that thyroxine is its precursor (10). It is possible to think that there are different conditions for the transformation of thyroxine to tri-iodothyronine in various organs and tissues. The onset and duration of the hormonal action is more rapid after the administration of tri-iodothyronine than after thyroxine (2, 15, 16). All this must be kept in mind when evaluating the results of quantitative tests of their activity. The present test merely shows a situation picture after three days treatment. In other test conditions the result would be different. There seems, accordingly to be good reason to go on with the experiments, e.g. by recording the rate of beat for several days after a single injection of tri-iodothyronine and thyroxine. In any case, the result of the present study assumes the same direction as previous reports. It indicates that the rate

of the isolated spontaneously beating rat auricle can be used as a quantitative indicator of the biological action of thyroxine and related substances.

#### SUMMARY

The biological activity of l-tri-iodothyronine and l-thyroxine was determined on the basis of rate acceleration in isolated rat auricle after three days treatment with these compounds. A typical dose-effect relationship was found. On the weight basis, in biological action, a ratio of 10:1 in favor of tri-iodothyronine was found.

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## DUODENAL SECRETION AND DYE ELIMINATION

by

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The elimination of dyes by gastric and pancreatic glands has been studied for various reasons (9, 12). Because of their widely varying chemical configuration and many kinds of physical properties, they may provide a clue to the structure and secretory mechanism of the glands; this in the case that certain regularities can be found in the character of the dyestuffs which do and do not appear in secretion of the glands. The chemical structure of the dyes used, however, has failed to throw any light on this question (2, 3, 11, 13). Studies of gastric pouches have shown that there is no relation between the oil-water distribution coefficients of various dyes and their secretability. Likewise there is no connection between their diffusion coefficients and their secretability (7). With certain reservations, it seems evident that only the dyestuffs whose chromogen can be positively charged either in the oxidized or reduced form appear in gastric juice (6, 11, 12). As for the pancreatic juice, it appears that the chromogen must have the form of an anion in the oxidized or reduced form in order to be secreted (6, 12). In both cases the only obvious regularity is that connected with the electrochemical properties of the dye (12).

Duodenal glands have their special characteristics distinguishing them from both the gastric and the pancreatic glands. To our knowledge there is no information available concerning dyestuff secretion by the duodenal glands. This paper describes an attempt to throw light on the functional characteristics of the duodenal

TABLE 1

LIST OF DYES USED IN THE PRESENT STUDY. CHEMICAL CLASSIFICATION AND THEIR APPEARANCE IN GASTRIC AND PANCREATIC JUICE ACCORDING TO PREVIOUS REPORTS (2, 3, 6, 7, 13)

Dye	Chemical Classification	Appli- cation Class	Location of Chromogen	Appear- ance in Gastric Juice	Appear- ance in Pan- creatic Juice
Neutral Red	Azine	Basic	In Cation	+	
Safranin T	Azine	Basic		+	
Methylene Blue	Thiazine	Basic	In Cation when Oxidized, both at pH 8.4 Re- duced	+	+
Malachite Green	Triphenylmethane	Basic	In Cation	+	
Basic Fuchsin	Triphenylmethane	Basic			+
Methyl Violet	Triphenylmethane	Basic	In Cation	—	+
Gentian Violet	Triphenylmethane	Basic	In Cation when Oxidized, both when Reduced		
Eosin	Pyronine	Acid		—	+
Methyl Orange	Azo	Acid	In Anion	—	+
Congo Red	Azo	Acid	In Anion	—	
Trypan Blue	Dis Azo	Acid	In Anion	—	

glands by administration to a dog of dyestuffs, most of which are known from previous studies to be secretable by either the gastric glands or the pancreas.

#### METHODS AND RESULTS

Four female dogs with duodenal pouches were used as the experimental animals. The first part of the duodenum, from the pylorus to the entrance of the common bile duct, was isolated and transplanted through the abdominal wall so that pure duodenal juice could be collected. The operative procedures have been described in previous papers (5, 10). The dyestuffs of the present study are listed in Table 1. From 5 to 20 cc of 0.2 or 0.5 per cent water solution of the respective dyestuffs was injected intravenously. The solutions were made isotonic by adding suitable amounts of 50 per cent glucose solution immediately before the injection. Samples

TABLE 2

LIST OF DYES USED IN THE PRESENT STUDY. NONE OF THEM WAS DETECTED IN THE DUODENAL JUICE

Dye	Manu- factured by	Dose of Dye (mg per kg of body weight)			
		Dog I (weight 15 kg)	Dog II (weight 12.5 kg)	Dog III (weight 10 kg)	Dog IV (weight 7.5 kg)
Neutral Red .....	Merck	1.3	1.6	2.0	2.7
Safranin T .....	Merck	3.3	4.0		4.7
					6.7
Methylene Blue ....	Bayer	1.3	1.6	2.0	2.7
Malachite Green ....	Merck	1.3	1.6		2.7
		1.3			
Basic Fuchsin .....	Bayer	1.3	1.6	2.0	2.7
Methyl Violet .....	Merck	1.3	1.6		2.7
		1.3			
Gentian Violet ....	Bayer	3.3	4.0		3.3
			4.0		
Eosin .....	Bayer	1.3	1.6		2.7
			3.2		
Methyl Orange ....	Bayer	1.3	1.6	2.0	2.7
Congo Red .....	Bayer	1.3	1.6		2.7
					2.7
Trypan Blue .....	Judex	3.3	4.0		6.7
			4.0		

of duodenal juice were collected over a period of 4  $\frac{1}{2}$  to 8 hrs (average 6 hrs) after the injection. The samples were subjected to visual examination both in their native form and after the change in pH made by adding 0.1-n sodium hydroxide or 0.1-n hydrochloric acid. Blood samples were also taken from  $\frac{1}{2}$  to 2 hrs after the injection of the dyestuffs. Distinct colouring was regularly observed in the plasma. Control experiments showed that *in vitro* the adding of 1 drop (0.025 cc) of the 0.2 per cent dye to 1.5 cc of duodenal juice always gave a colour similar to the original dye solution; the concentration of the dye was 0.03 mg%. When this mixture was diluted with saline the colour of the dye was clearly perceptible in all cases after tenfold dilution (0.003 mg%) and in most cases after hundredfold dilution (0.0003 mg%).

The dogs tolerated the dye injections relatively well. Only neutral red caused restlessness immediately after the injection and

redness and swelling in the muzzle. Anorrexia was common in the days after the experiments. One of the dogs died during the tests and therefore the series is incomplete for that case. Occasionally, dye was observed in the faeces; e.g. after eosine and methyl violet injection. No systematic observations were made in this respect. After the last test, three of the dogs were necropsied. The renal cortex and mesenteric lymph nodes were bluish green.

The experiments with all the eleven dyestuffs were repeated four times. Only one injection was given per day. Table 2 shows the names and quantities of the dyes administered and the weights of the dogs. The results of all the tests were the same: in no case was the duodenal juice coloured after the injection of a dye.

#### DISCUSSION

The number of dyes studied is limited. They include, however, representative types of the dyestuffs that have been shown in previous studies to be secreted by either the gastric or the pancreatic glands or both. It is therefore interesting to note that none of these dyes could be detected in the duodenal juice.

The appearance of certain dyes in the faeces can be explained not only by their elimination in gastric or pancreatic juice but also by their secretion by the liver in the bile (1, 8). The dye concentration found in pancreatic juice has been very low according to Ingraham & Visscher (6); in most cases less than 1 per cent of the injected concentration in the blood. If the dose of an injected dye is 1 mg per kg of body weight, and if plasma represents as much as say 5 per cent of the body weight, then the concentration of the dye in plasma, after its distribution in the circulation, will be 2 mg %. One per cent of this concentration would mean 0.02 mg % of dye in the pancreatic juice. If the dyes appear in duodenal juice in concentrations equal to those found in the pancreatic juice, the example above can be applied to the duodenal juice. On the basis of preliminary experiments *in vitro*, a dyestuff concentration of 0.02 to 0.002 mg % in the duodenal juice is clearly perceptible to the naked eye. The relative dye concentrations detected in the gastric juice were generally much higher (6).

That none of the injected dyestuffs was detected in the duodenal juice is a rather unexpected observation, unexpected mainly because

of the close resemblance between the duodenal juice and certain properties of the pancreatic secretion. Both secretions are alkaline; the alkalinity arises from the bicarbonate content. If the electrochemical properties of the injected material were the decisive factor regulating the passage and elimination of certain chromogens through various cellular barriers, then the duodenal glands might be expected to behave in this respect more like the pancreatic glands. Our results lend no support to such a concept. It would appear that some other, still more selective mechanism must be considered. With the technique employed in the present study, no information as to the nature of such a mechanism can be suggested. In light of some recent studies on the specific detoxication function of both the intestinal and the gastric mucosa (4) which add to our knowledge of the function of these elements, it might be permissible to speculate on new metabolic activities in the area. Further studies might be able to elucidate the role of such factors.

## SUMMARY

Eleven dyes of various chemical and physical properties were injected intravenously into dogs prepared with a duodenal pouch. The dose was from 1.3 to 6.7 mg per kg of body weight. In no case was the dye observed to appear in the duodenal juice although nine of the dyes were known to be secretable by either the gastric or the pancreatic glands.

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## EFFECT OF ADRENALINE AND NORADRENALINE ON THE ISOLATED RAT AURICLE IN VARIOUS STATES OF THYROID ACTION

by

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Both adrenaline and noradrenaline (arterenol) have a positive chronotropic and inotropic effect on the heart muscle, especially when the experiments are performed on a partly or completely isolated preparation (1, 3—5, 7, 9—12, 18, 21, 22, 25, 26, 29, 30). *In situ* the effect of noradrenaline is somewhat dissimilar because of the reflex bradycardia caused by a rise in blood pressure (2, 8). Sensitivity of the heart muscle to various stimulating agents differs according to the conditions. The normal activity of heart muscle presupposes the presence of thyroid action. A thyrotoxic heart has been noted to be more susceptible to adrenaline than a normal or hypothyroid heart (6, 20, 24, 27, 32, 33). Numerous comparative studies have dealt with the action of adrenaline and noradrenaline on normal heart muscle (1, 5, 9, 11, 12, 22, 26, 30).

In the present study, hypothyroid, normal and hyperthyroid isolated rat auricles have been subjected to the action of increasing doses of adrenaline and noradrenaline in order to see if there is any difference in the reactions of the various preparations to the substances.

### MATERIAL AND METHODS

The experiments were performed on 30 isolated spontaneously beating right auricles of the rat. Ten of them originated from normal animals, 10 from hypothyroid rats which received 400  $\mu$ c



of radioactive iodine 46 to 54 days before the test (15, 28), and ten from hyperthyroid rats treated with l-tri-iodothyronine (Glaxo) for a period of three days before the experiment; the daily dose was 0.3 mg (16). All the experimental animals were female albino rats weighing on an average 184 g (range 165 to 220 g). The rat was stunned with a blow. The right auricle was isolated and the isometrical mechanogram registered as described previously (13). The stretch applied to the preparation during recording was constant throughout the test. All the experiments were performed at 37°C. A stream of pure oxygen was forced through alkaline Locke's solution in the isolation chamber. The volume of the solution was 50 cc. dl-Adrenaline («Orion» Adrenal) and l-noradrenaline («Orion» Nor-Adrenal) were added to Locke's solution in increasing concentrations after two initial recordings. This was performed so that the concentration of the substance after the additions was as follows:  $1 \times 10^{-8}$ ,  $3 \times 10^{-8}$ ,  $5 \times 10^{-8}$ ,  $1 \times 10^{-7}$ ,  $3 \times 10^{-7}$ ,  $5 \times 10^{-7}$ ,  $1 \times 10^{-6}$  and  $1 \times 10^{-5}$ . The recordings were made one minute after adding the substance in question. The whole procedure was completed in 10 to 12 minutes. Both adrenaline and noradrenaline tests were carried out on all the preparations. The adrenaline or the noradrenaline series was performed first in alternative cases. After this the preparation was washed several times with Locke's solution and the following experiment was started after an interval of one hour. All the experiments were performed in the middle of summer. Therefore the effect of possible seasonal variations in the sensibility of preparation's to adrenaline and noradrenaline were excluded (19).

#### RESULTS

The hypothyroid state caused by radioactive iodine and the hyperthyroid state as a result of tri-iodothyronine treatment were indicated by the initial beating rate at 37°C. As Table 1 and Figure 1 show, there is no doubt that the treatment was effective in both cases (c.f. 15, 16).

The effect of adrenaline and noradrenaline on the rate of beat is seen in Figure 1 and the corresponding cycle lengths are listed in Table 1. In all the preparations a powerful positive chronotropic effect was obtained. The absolute figure of acceleration in rate (per

TABLE 1

EFFECT OF ADRENALINE AND NORADRENALINE ON THE CYCLE LENGTH, DURATION OF CONTRACTION PERIOD AND CONTRACTION FORCE OF THE ISOLATED HYPOTHYROID, NORMAL AND HYPERTHYROID RAT AURICLE

Concentration of the Respective Substance	Adrenaline Series				Noradrenaline Series			
	Cycle Length (msec)	Contraction Period		Contraction Force (arb. unit)	Cycle Length (msec)	Contraction Period		Contraction Force (arb. unit)
		msec	% of Cycle			msec	% of Cycle	
0	277 ± 9	36 ± 1.0	13	100 ± 0	268 ± 11	33 ± 1.1	12	100 ± 0
1 × 10 <sup>-8</sup>	254 ± 16	35 ± 1.1	14	117 ± 5	263 ± 15	33 ± 1.1	13	101 ± 1
3 × 10 <sup>-8</sup>	241 ± 17	36 ± 1.2	15	120 ± 7	259 ± 18	33 ± 1.1	13	103 ± 2
5 × 10 <sup>-8</sup>	239 ± 18	36 ± 0.8	15	124 ± 8	257 ± 19	34 ± 1.2	13	106 ± 4
1 × 10 <sup>-7</sup>	233 ± 13	35 ± 1.0	15	125 ± 15	255 ± 24	33 ± 1.7	13	105 ± 3
3 × 10 <sup>-7</sup>	194 ± 9	36 ± 1.3	19	171 ± 16	188 ± 5	33 ± 1.1	18	139 ± 7
5 × 10 <sup>-7</sup>	190 ± 6	35 ± 1.1	19	165 ± 15	187 ± 5	33 ± 1.0	18	130 ± 6
1 × 10 <sup>-6</sup>	184 ± 5	35 ± 1.0	19	180 ± 15	181 ± 4	32 ± 0.8	18	140 ± 7
1 × 10 <sup>-5</sup>	191 ± 6	36 ± 1.2	19	206 ± 17	186 ± 6	33 ± 1.0	18	165 ± 10
0	164 ± 4	24 ± 0.3	14	100 ± 0	174 ± 4	26 ± 0.5	15	100 ± 0
1 × 10 <sup>-8</sup>	149 ± 2	24 ± 0.3	16	108 ± 3	149 ± 4	26 ± 0.5	17	104 ± 3
3 × 10 <sup>-8</sup>	139 ± 2	24 ± 0.3	17	111 ± 4	142 ± 4	25 ± 0.4	18	101 ± 4
5 × 10 <sup>-8</sup>	134 ± 2	24 ± 0.3	18	110 ± 5	140 ± 3	26 ± 0.3	19	104 ± 4
1 × 10 <sup>-7</sup>	134 ± 4	23 ± 0.5	17	112 ± 6	133 ± 4	26 ± 0.5	20	99 ± 3
3 × 10 <sup>-7</sup>	122 ± 1	24 ± 0.1	20	139 ± 9	123 ± 2	25 ± 0.5	20	105 ± 5
5 × 10 <sup>-7</sup>	122 ± 1	25 ± 0.5	20	138 ± 12	123 ± 2	25 ± 0.4	20	109 ± 3
1 × 10 <sup>-6</sup>	122 ± 1	24 ± 0.3	20	130 ± 7	122 ± 2	25 ± 0.6	21	111 ± 5
1 × 10 <sup>-5</sup>	129 ± 2	24 ± 0.4	19	132 ± 7	121 ± 1	26 ± 0.6	22	120 ± 5
0	129 ± 1	21 ± 0.4	16	100 ± 0	127 ± 2	21 ± 0.4	17	100 ± 0
1 × 10 <sup>-8</sup>	114 ± 2	21 ± 0.3	18	113 ± 3	119 ± 2	21 ± 0.4	18	102 ± 3
3 × 10 <sup>-8</sup>	112 ± 2	21 ± 0.4	19	110 ± 4	114 ± 2	21 ± 0.2	18	104 ± 4
5 × 10 <sup>-8</sup>	111 ± 2	21 ± 0.3	19	108 ± 5	113 ± 2	21 ± 0.4	19	104 ± 4
1 × 10 <sup>-7</sup>	110 ± 2	21 ± 0.4	19	111 ± 4	112 ± 2	21 ± 0.4	19	103 ± 4
3 × 10 <sup>-7</sup>	108 ± 1	21 ± 0.3	19	125 ± 3	108 ± 1	21 ± 0.4	20	110 ± 7
5 × 10 <sup>-7</sup>	108 ± 1	21 ± 0.3	19	131 ± 5	107 ± 2	21 ± 0.4	20	109 ± 8
1 × 10 <sup>-6</sup>	107 ± 1	22 ± 0.4	20	122 ± 5	106 ± 1	21 ± 0.2	20	110 ± 8
1 × 10 <sup>-5</sup>	107 ± 1	21 ± 0.4	20	121 ± 3	105 ± 1	21 ± 0.4	20	114 ± 8

minute) after the addition of small doses showed a smaller increase in hypothyroid preparations than in the normal and hyperthyroid ones. When big doses were used, these figures did not show any essential differences. The observed relative maximum acceleration of rate in normal auricles was 34 to 44 per cent of the initial value.

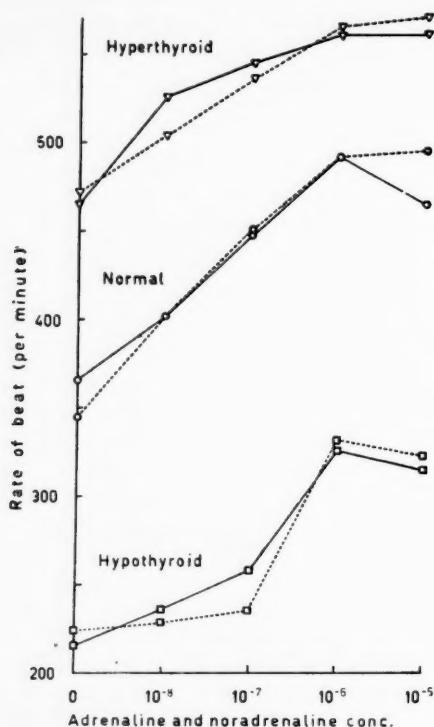


Fig. 1. — The beating rate of various auricles in adrenaline (—) and noradrenaline (.....) series. Abscissa: logarithmic scale.

In hypothyroid preparations the respective figure was 50 per cent, in hyperthyroid auricles only 20 per cent. The rate curves of all the preparations are similar both after adrenaline and noradrenaline treatment. No essential differences are to be seen.

The contraction force increased in all the preparations when the substances in question were added as Figure 2 and Table 1 show. The maximum increase was relatively highest in the hypothyroid preparations. There were no significant differences between

the normal and the hyperthyroid auricles. Adrenaline had a more powerful effect than noradrenaline on the contraction force of all the preparations.

The duration of the contraction phase of an isometric contraction did not change with alterations in cycle length (rate of beat) and contraction force, as Table 1 indicates. As a result, the ratio of

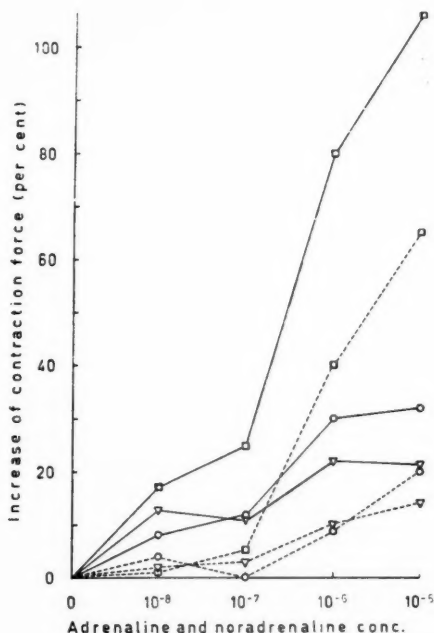


Fig. 2. — Increase in contraction force of various preparations caused by adrenaline (—) and noradrenaline (.....). □ Hypothyroid, ○ normal and △ hyperthyroid auricles. Abscissa: logarithmic scale.

contraction phase to cycle length increased as the rate accelerated with increased doses of adrenaline and noradrenaline. In this respect there were no differences between the various preparations.

#### DISCUSSION

Increasing doses of adrenaline and noradrenaline had an increasing effect on the beating rate and contraction force of auricular preparations. Too great an importance should not be attributed to the dose-effect relationship, as such. By the time an additional

dose of the substance was added, the previous dose had apparently been partially destroyed in the alkaline solution in the presence of oxygen and absence of plasma (8, 17); furthermore, adrenaline is to be regarded as a potential drug (34). However, all the preparations were examined under similar test conditions. Therefore the results with the various preparations are comparable and serve the purpose of the study.

The positive chronotropic effect of adrenaline and noradrenaline are in agreement with previous reports on the heart muscle in isolated state. The differences between the effects of adrenaline and noradrenaline found in the present study were not convincing. Burn & Hutcheon (4) established on the isolated cat heart that the effects of adrenaline (0.1  $\mu\text{g}$ ) and noradrenaline (0.26  $\mu\text{g}$ ) were almost identical on the amplitude, though noradrenaline had less effect on the rate. Lands & Howard (22) found adrenaline to be more effective than l-noreadrenaline in increasing the amplitude and rate of contraction in the perfused frog heart and isolated sino-auricular preparation of tortoise. When the isolated auricle or perfused heart of a rabbit was used, adrenaline was noted to be less effective than l-noradrenaline. The activity ratio between adrenaline and noradrenaline on heart rate depends on the test animal used.

Many writers conclude that hyperthyroid heart muscle is more sensitive than the normal to adrenaline (6, 20, 24, 27, 32) and that the hypothyroid heart shows decreased sensitivity to it (33). As for the beating rate in the present study, the percentage of the maximum acceleration was highest in the case of hypothyroid preparations and lowest in hyperthyroid auricles. To small adrenaline and noradrenaline doses, the rate of hypothyroid auricles seemed to react more weakly than the rate of normal controls. In such cases the action of noradrenaline was weaker than that of adrenaline. With the addition of big doses of both compounds, there were no marked differences.

Noradrenaline was less powerful than adrenaline in strengthening the contraction force. This is in agreement with Bakera's (3) observation on human foetal hearts. According to Gazes *et al.* (10), the increment in the contractile force in unanaesthetized trained dogs after administration of l-noradrenaline was of similar magnitude to that produced by l-adrenaline. They present the effect on the contractile force of the heart of l-noradrenaline as an explanation

of the high recovery rate observed in the treatment of shock accompanying myocardial infarction.

Both adrenaline and noradrenaline had a relatively more marked effect on hypothyroid preparations than on normal and hyperthyroid auricles. The contraction force of a hypothyroid rat auricle does not differ markedly at 37°C from that of a normal one (15). However this does not mean that the work output per minute after adrenaline or noradrenaline treatment is greater in hypothyroid than in normal or hyperthyroid preparations because of the slower rate.

Hilton (12) on the basis of tests on isolated rabbit heart, notes that the duration of inotropic response in the adrenaline series showed a longer duration of drug action than did the hearts treated with identical doses of noradrenaline. Walker & Lourie (35) came to similar conclusions.

Comparison of various reports and the results of the present study shows that the concept of sensitivity to adrenaline or noradrenaline must be clearly defined before it is used. The present experiment indicates that the reaction of various preparations changed with the size of the dose, was different as regards the beating rate and contraction force and differed according to whether absolute or relative changes were considered. Moreover there can be dissimilarities when the effect is evaluated on the basis of the start of contractile activity in preparations which do not beat spontaneously (20, 23).

Although the beating rate of all preparations increased markedly as a result of the effect of adrenaline and noradrenaline, the duration of the contraction phase did not show any tendency to shorten. When the rate of an isolated auricle increases with rising temperature, the contraction period shortens (13, 14, 15). When the heart rate *in situ* is accelerated by adrenaline, not only diastole but also systole shortens, independent of the heart rate. This is supposed to be a specific adrenergic effect (31).

#### SUMMARY

The effect of adrenaline and noradrenaline on the mechanogram of isolated spontaneously beating auricular preparation of hypothyroid, normal and hyperthyroid rats was studied.

Both the substances had a practically similar effect on the beating rate of the various auricles. The relative maximum effect was highest in hypothyroid preparations. Hypothyroid auricles were less sensitive to small doses than the normal and hyperthyroid auricles, as regards the absolute increase in rate.

The contraction force increased more after the administration of adrenaline than with noradrenaline. The relative increase in contraction force was highest in the hypothyroid preparations.

Although the contraction rate and force increased, the duration of the contraction phase of isometrical contraction remained unchanged in all the preparations.

The concept of sensitivity to adrenaline or noradrenaline is to be defined before it is used.

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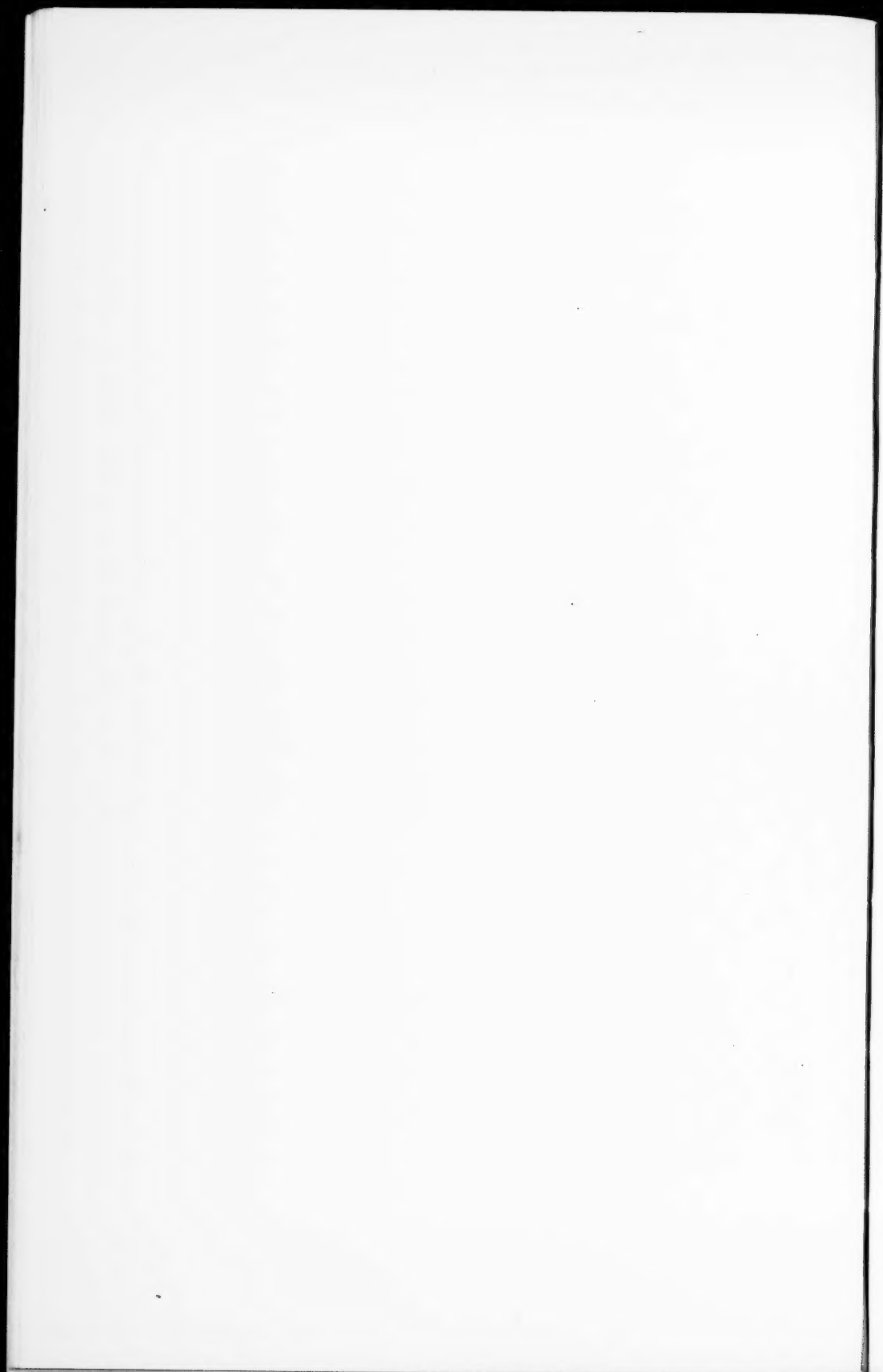


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### ERRATUM

*Ann. Med. Exper. Fenn.*, Vol. 33., Fasc. 1—2, 1955.  
"Hemophilus Pertussis, Sensitivity to Eight Anti-  
biotics and Sulphathiazole", by Simo Virtanen.

Page 54, second line of the summary: "resistant"  
should read "sensitive" and the fourth line should  
not include "penicillin".



## STUDIES ON DETOXICATION MECHANISMS<sup>1</sup>

### V

#### EFFECT OF THYROXINE ON GLUCURONIDE DETOXICATION SYNTHESIS OF VARIOUS ORGANS

by

K. J. V. HARTIALA, LEO HIRVONEN and A. KASSINEN

(Received for publication December 19, 1955)

Following the observation that glucuronide detoxication synthesis is to a large extent carried out also by the mucous membrane of the alimentary canal (11), it was a matter of some urgency to elucidate the relationship between the detoxication properties of the liver and the intestinal canal under various conditions.

The present study was undertaken in order to find out whether factors which affect the metabolic processes in the organism show any difference in their effect on the detoxication mechanism. This series includes studies in which the animals were treated with thyroxine before the various organs were studied for their detoxication capacity.

#### MATERIAL AND METHODS

The studies were carried out on 15 male rabbits weighing 1.8 to 2.7 kg (average 2.1 kg). Nine of them received dl-thyroxine (British Drug) subcutaneously in three or four successive days, the whole dose being 4 mg. The remaining 6 rabbits served as controls. Six control animals were considered sufficient because additional data were available from 35 control rabbits studied under similar

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<sup>1</sup> This study was aided by grants from the Sigrid Jusélius Foundation.

test conditions in previous experiments (11). Since it is not yet known whether sex makes any difference to the detoxication capacity, all the animals used were of the same sex.

The animals were killed by a blow on the head, the organs removed immediately and placed in ice-cold Ringer's solution. The detoxication synthesis was followed by using the coupling of o-aminophenol to the corresponding glucuronide, taking this as a measure of the detoxication capacity. The method has been described in detail previously (12, 16).

No direct observations were carried out to establish the effect of the thyroxine treatment on the metabolic processes or circulatory system. According to previous experiments (7) the thyroxine dosage used results in a clear acceleration of the spontaneous beating rate in isolated rabbit auricle. As many of these animals were also used for similar studies according to the technique published previously (13), the recorded beating rate was useful as an indication of thyroxine action.

Fechner's formula was used in the calculation of standard errors (5).

#### RESULTS AND DISCUSSION

The mean rate of beat in six isolated auricles at 35°C was  $234 \pm 11$  and at 40°C  $323 \pm 13$  per minute. This means an average acceleration of about 30 per cent from that found in eight normal auricles in which the corresponding mean rates were  $184 \pm 10$  and  $247 \pm 12$  per minute. The result is in agreement with observations by other authors (9, 17).

The detoxication capacity of the various organs in the experiments is listed in Table 1. The control results concur with previous studies made in this laboratory on untreated rabbits (11) in which the corresponding figures for glucuronide coupled per mg dry weight of liver tissue was 123  $\gamma$  (range 38 to 236  $\gamma$ ).

As Table 1 shows, the average value for liver in the thyroxinized series was twice as high as in the control animals, the difference ( $125 \pm 31$ ) being statistically significant. Another observation is that the pyloric figures showed no marked difference from the control experiments. The duodenal figures were only 50 per cent of the control values. The difference ( $108 \pm 41$ ) is statistically only probable. It is, however, interesting to note that if there is any

TABLE 1

EFFECT OF THYROXINE ON THE DETOXICATION MECHANISM. O-AMINOPHENOLGLUCURONIDE CONJUGATED BY VARIOUS ORGANS ( $\gamma$  PER mg TISSUE DRY WEIGHT).  
TIME 90 MINUTES

No.	Liver		Mucous Membrane						Kidney	
			Pylorus		Duodenum		Ileum			
	Thyrox.	Control	Thyrox.	Control	Thyrox.	Control	Thyrox.	Control	Thyrox.	Control
1	330	194	—	—	131	269	154	—	31	—
2	259	126	120	118	123	300	—	—	11	11
3	173	93	—	89	119	262	—	—	10	—
4	282	106	8	181	98	171	—	306	—	30
5	220	83	60	116	59	215	—	156	—	16
6	247	56	116	47	75	32	—	79	—	—
7	318		—		118		98		35	
8	211		129		156		166		10	
9	74		—		22		33		—	
Mean	235	110	87	110	100	208	113	180	19	19
S.E.	25	18			14	38				

difference in the duodenal detoxication behaviour under thyroxine, this change is in a direction opposite to that where the liver is concerned.

These experiments indicate that thyroxine treatment increases the liver's capacity to synthesize glucuronides. The glucuronide synthesis is a chemical process associated with increased oxygen consumption (16). The augmentation of the oxidative processes and oxygen consumption of various tissues removed from thyroxine-treated animals is demonstrated by earlier observations (2, 4, 18). In fact, the onset of thyroxine action is slow also *in vivo*. Added *in vitro* to the surrounding medium it does not show any immediate effect.

Previous studies have also shown that thyroid hormone or thyroxine does not have the same effect on the various organs. The cardiac muscle responds to thyroid treatment with increased oxygen consumption, whereas intestinal muscle does not show similar behaviour (18, 21). As for the liver and kidney tissue, the information is contradictory (3, 8). No data are available as to the effect of thyroxine on the oxygen consumption of intestinal mucous elements.

The role of the liver in the metabolism of thyroxine is of particular interest. Several authors have recently demonstrated that thyroxine is concentrated by the liver and excreted in the bile (1, 6, 10, 14, 15). It is also known that thyroxine is eliminated as glucuronide, a synthesis that is obviously carried out by the liver (19, 20). It may be that these latter observations explain the stimulating effect of thyroxine on the glucuronide synthesis capacity in our experiments.

#### SUMMARY

Studies were made of the glucuronide synthesis of various rabbit organs after 3 to 4 days thyroxine treatment (total dose 4 mg). It was found that:

1) The capacity of the liver to synthesize glucuronide *in vitro* in the presence of o-aminophenol was doubled.

2) The detoxication capacity of intestinal mucous membrane was not affected in the same manner. If there was any effect, it was a depressive one on the duodenal glucuronide synthesis.

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## STUDIES ON DETOXICATION MECHANISMS<sup>1</sup>

### VI

#### EFFECT OF RADIOACTIVE IODINE ON THE GLUCURONIDE SYNTHESIS OF VARIOUS ORGANS

by

K. J. V. HARTIALA and LEO HIRVONEN

(Received for publication December 19, 1955)

In connection with studies on the effect of thyroxine on glucuronide formation the observation was made that the detoxication synthesis by the liver increased in thyroxine-treated rabbit (9). The present study was undertaken in order to find out whether hypothyroid animals show a different behaviour as regards to their detoxication capacity.

#### MATERIAL AND METHODS

The experiments were performed on 26 albino rats. Thirteen of them received subcutaneously 300 microcuries of radioactive iodine ( $I^{131}$ ) as a single dose 45 to 56 days before the test. This kind of thyroidectomy induced by the internal ionizing radiation of  $I^{131}$  has several advantages over surgical thyroidectomy. The factors with a bearing on the effect of radioactive iodine have been well established by several investigators (3—8, 11, 14). Thirteen untreated rats were used as controls. There were no restrictions on the iodine content of the diet of the animals. The rats were killed with a blow on the head. The thyroids of several animals were removed, fixed in formalin and prepared for microscopic examina-

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<sup>1</sup> This study was aided by grants from the Sigrid Jusélius Foundation

TABLE 1

THE EXPERIMENTAL ANIMALS TREATED WITH RADIOACTIVE IODINE (300 micro-curies of  $I^{131}$ ). INDICATIONS OF THE EFFECTIVENESS OF THE TREATMENT

Sex	No.	Weight of Rats (g)				Beating Rate of Isolated Auricle at 37°C
		Before Injection	30 Days Later	Before Test (40 to 56 Days)	Gain in Weight	
Female	1	145	160	170	25	312
	2	150	170	180	30	313
	3	160	180	190	30	359
	4	170	180	180	10	308
	5	220	210	210	-10	236
	6	175	220	225	50	402
	7	160	170	180	20	320
	8	170	180	190	20	385
	Mean	169	184	191	22	329
Male	1	180	240	255	75	364
	2	205	270	275	70	358
	3	165	225	235	70	436
	4	165	260	295	130	—
	5	170	240	245	75	407
	Mean	177	247	261	84	391

tion. At the same time the animals were used in the mechanogram observations of the isolated auricle of hypothyroid rat (10). Thus the spontaneous beating rate of the auricles was available as an additional indication of the effectiveness of  $I^{131}$  treatment.

#### RESULTS

Table 1 gives the weights of the experimental animals before the injection of  $I^{131}$  and before the test, and the auricular beating rate at 37°C. The mean weight of the female rats was only 22 g higher before the test than at the time of injection. The corresponding weight increase of the male rats was 84 g. Impairment in the ability to gain weight as a result of  $I^{131}$  treatment has been established by Maloof *et al.* (11). The average weight of the female control rats was 188 g (range 160 to 225 g) and that of the males 266 g (range 215 to 300 g).

TABLE 2

EFFECT OF RADIOACTIVE IODINE ON THE DETOXICATION MECHANISM. O-AMINO-PHENOL-GLUCURONIDE CONJUGATED BY VARIOUS ORGANS ( $\gamma$  PER mg TISSUE DRY WEIGHT). TIME 90 MINUTES

Sex	No.	Liver		Mucous Membrane						Kidney	
		$I^{131}$	Con-trol	Pylorus		Duodenum		Ileum		$I^{131}$	Con-trol
				$I^{131}$	Con-trol	$I^{131}$	Con-trol	$I^{131}$	Con-trol		
Female	1	25	62	—	68	129	234	76	32	72	142
	2	26	83	27	110	130	298	24	183	88	—
	3	9	110	54	108	200	160	—	20	35	250
	4	12	—	82	62	458	80	17	14	90	200
	5	—	75	29	10	76	36	15	139	116	100
	6	50	54	—	67	190	53	115	23	71	138
	7	11	65	72	—	344	133	23	42	31	25
	8	78	66	122	97	255	19	148	—	148	56
	Mean	30	74	64	87	223	127	60	65	81	130
Male	1	21	83	53	118	67	80	79	38	101	85
	2	14	33	25	—	—	—	—	—	—	34
	3	35	21	41	54	—	44	80	41	51	73
	4	8	8	68	18	172	278	—	—	54	62
	5	90	27	66	117	123	552	66	162	176	125
	Mean	34	34	51	77	91	239	75	80	96	76

The average auricular beating rate of the  $I^{131}$ -treated female group was 329 per minute and that of the corresponding control group 375 per minute. The average rate of a collected material of 34 untreated rat auricles under similar test conditions was  $369 \pm 5$  per minute. In six cases out of eight, in the present test, the rate of beat was below this. In the auricles of male rats treated with  $I^{131}$ , there was no retardation in the beating rate.

In the histological samples where available, the following changes were observed:<sup>1</sup> partial or total lack of colloid, cellular hypertrophy, increase of fibrous tissue and swelling in the walls of the arteries. Some normal follicles were observed among the changed ones especially in the periphery of the gland (c.f. 3). Figure 1 shows histological changes observed after  $I^{131}$  treatment

<sup>1</sup> The authors are indebted to Docent C. v. Numers for his kind assistance in the interpretation of the histological findings.

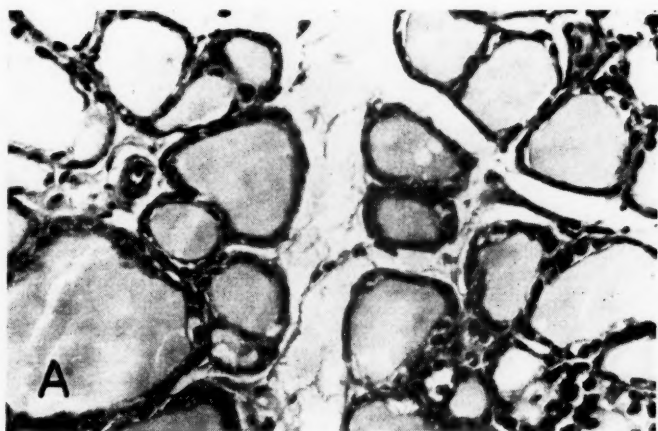


Fig. 1. — A. Thyroid gland from a control rat. B. Thyroid gland from a rat (♂ No. 3) 50 days after the administration of 300 microcuries of radioactive iodine. Observe the decrease of colloid and the hypertrophy of cells. C. Thyroid from a rat (♀ No. 4) 52 days after administration of 300 microcuries of  $I^{131}$ . Note the marked degree of lack of colloid, the cellular hypertrophy and the increase of fibrous tissue. — (van Gieson;  $\times 700$ ).

together with the thyroid tissue of a normal control rat. The findings are in accordance with previous reports (3, 5, 11). The histological samples were similar whether the auricular beating rate was retarded or not.

The results of the detoxication tests are listed in Table 2. It can be seen that the glucuronide synthesis of the liver was clearly diminished in the  $I^{131}$ -treated female series. The function of other organs known to possess the ability to perform glucuronide synthesis was not affected in a similar manner. It is of particular interest that under conditions where the liver had a very minute capacity to perform glucuronide synthesis, the intestinal, mainly the duodenal, capacity was very high. In the group of male rats, diminishing glucuronide synthesis by the liver was observed only in two out of five cases.

There was a clear parallelism between the depression of the detoxication synthesis of the liver and the beating rate of the isolated auricle. Various criteria indicate that in the present material male rats were less sensitive to  $I^{131}$  treatment than the females. Although there are observations that show functional changes in the thyroid gland caused by  $I^{131}$  without concurrent histological



Fig. 1 B.

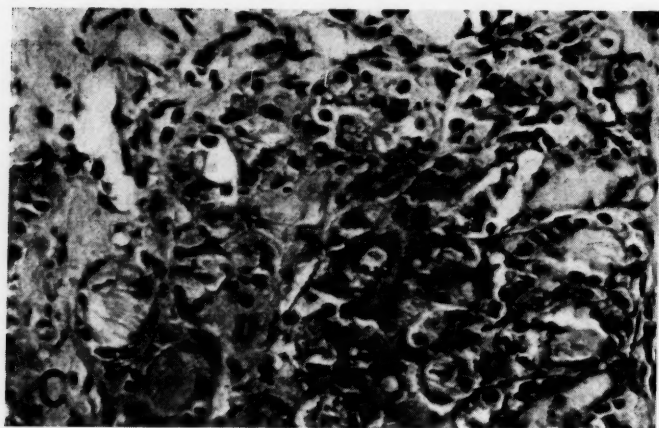


Fig. 1 C.

changes (11), in the present test there were cases in which the histological changes were not followed by functional changes in the periphery. The present test, however, is to be considered as a preliminary one. It provides interesting findings for further studies.

The results of the present study are in striking contrast to those observed in the rabbits treated with thyroxine (9). They may be taken as an indication that the detoxication function is affected by factors modifying the general metabolism processes. The effects

of increased thyroid hormone and the hypothyroid condition on the glucuronide synthesis can, however, hardly be attributed to any general changes in aerobic processes. According to Anselmino *et al.* (1) thyroxine does not increase the oxygen consumption of the liver. Dresel's (2) tests showed an opposite result. As mentioned in our previous paper (9), we would tend to the view that the thyroid effects on the liver can be explained by the role of this organ among the mechanisms that control the amount of thyroid hormone in the general circulation. In these processes the glucuronide synthesis appears to be of significance (12, 13). That the excess amounts of the thyroid products and diminished function of the gland may lead the adaptive properties of the liver to increase or decrease its capacity to perform the glucuronide conjugation reactions is of particular interest. Taking this interpretation as correct, further study is called for in order to learn the mechanism associated with the condition of hypo- and hyperthyrosis in the organism.

As to the glucuronide synthesis of other organs, at least one interesting remark can be offered. Even though we are not inclined to claim any specific effect for thyroidectomy on the intestinal glucuronide synthesis, the fact remains that in conditions where the hepatic function is impaired, the capacity of mucous membranes to carry out such synthesis is still strong. In other words, the glucuronide synthesis in various organs is not dependent on the same factors.

#### SUMMARY

Studies were made of the glucuronide synthesis of various organs of rats injected 45 to 56 days earlier with 300 microcuries of  $I^{131}$ . It was found that:

The capacity of the liver to synthesize glucuronide *in vitro* in the presence of o-aminophenol was diminished more than 50 per cent.

The detoxication capacity of intestinal mucous membrane did not show parallel changes.

The changes observed are the opposite of those found after thyroxine treatment in rabbit.

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## STUDIES OF THE PERMEABILITY OF GUINEA PIG PLACENTA TO RADIOACTIVE IODINE AND THYROXINE INJECTED INTO A FOETUS<sup>1</sup>

by

HARRY LYBECK and LEO HIRVONEN

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According to Joliot *et al.* (7) radioactive iodine administered to a rabbit in the middle of pregnancy permeates the placenta more easily than radiothyroxine. In a previous study (6) the present writers have shown that thyroxine administered to a pregnant guinea pig hardly permeates the placenta in the first hours after its application although iodine is soon detected in the foetal blood. It has been supposed that there is a two-way passage of iodine and thyroxine through the placenta. To our knowledge no direct experiments have been performed in order to show the permeability of placenta to these substances injected into the foetus. The present study deals with the permeability of the guinea pig placenta to radioactive iodine and thyroxine in the foetus in the direction of the mother.

### METHODS

The experiments were performed in a manner essentially similar to those in which iodine and thyroxine were injected into mother animals (6). Eleven pregnant guinea pigs with their 33 foetuses served as the experimental animals. Five guinea pigs were used for studies with radioactive iodine ( $I^{131}$ ) and six received radioactive thyroxine (labeled with  $I^{131}$ ). The mother animal was anaesthetized with an intraperitoneal »Nembutal» injection. The abdominal

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<sup>1</sup> This study was aided by grants from the Sigrid Jusélius Foundation.

wall was incised. The uterine wall was incised also. A silk thread was fastened to the skin of a foetus. A long and fine syringe was applied subcutaneously. After the injection the thread was knotted around the syringe. When the syringe was drawn out the injection point was closed by the ligature. The incision hole of the uterine wall was then closed with a silk thread and the abdominal wall sutured in the usual way. The narcosis was continued until the end of the experiment. The following samples were collected: maternal blood by heart puncture, amniotic fluid, foetal blood by heart puncture, and maternal bile. The samples of the foetus which had received radioactive material were taken last. The liquid samples were dried and counted with an equipment consisting of an automatic sample changer for Geiger-Müller tubes, electronic scale of 8, counter and mechanical registers. The thyroxine labeled with  $I^{131}$  was freshly prepared and identified chromatographically (8). It was then dissolved in M/50  $Na_2CO_3$  solution.

#### RESULTS

The results of the radio-iodine experiments are listed in Table 1. Comparing the radioactivity of the foetal blood samples with those of maternal blood, a great difference can be found. On the basis of differences in weight, it can be concluded that in the maternal blood a much greater quantity of radio-iodine is needed to maintain a certain radioactivity than in the case of foetal blood. Significant radioactivity was also detected in the blood and amniotic fluid samples of intact foetuses, indicating the existence of a two-way passage of iodine through the guinea pig placenta. It may be possible, however, that foetal tissues have a greater affinity to iodine than those of the mother animal. In a previous study (6), in which iodine was injected into mother animals, more radioactivity was found in foetal than in maternal blood in many cases. This phenomenon can be explained by the disappearance of iodine from the maternal blood stream e.g. by iodine collection of the thyroid gland or by its excretion in urine.

Table 2 shows the results of radiothyroxine tests. The thyroxine dose on the radioactivity basis was 1 to 3 microcuries per test. This was less than the dosage used in iodine experiments. In the radiothyroxine used in the present study, apparently only one of the

TABLE 1

RADIOACTIVITY OF VARIOUS SAMPLES AFTER INJECTION OF RADIOACTIVE IODINE INTO ONE OF THE FOETUSES

Experiment No.	Duration of Test (hr)	Foetus No. <sup>1</sup>	Weight of Foetus (g)	Radioactivity (Counts per Min.) <sup>2</sup>			Weight of Pregnant Guinea Pig (g)	Iodine Dose (μc)
				Foetal Samples (1/4 cc)		Maternal Blood (1/4 cc)		
				Blood	Amniotic Fluid			
1	2 1/4	1	110	202	218	637	1025	10
		2	112	1282				
2	1	1	65	13	12	40	1000	5
		2	85	11	16			
		3	70	693				
3	1	1	70	219		778	945	10
		2	85	274				
		3	95	323				
		4	90	2412				
4	1	1	210	34	26	31	1050	7
		2		36				
		3		1901				
5	3	1	45	36	25	60	850	5
		2	50	32	20			
		3	50	2075				

<sup>1</sup> The numbers of the foetuses receiving radioactive iodine have been printed in bold type.

<sup>2</sup> The value of the background (19 to 22 c. p. m.) has been subtracted from each of the recorded radioactivity values.

iodine atoms was radioactive. Therefore the radioactive iodine represented only a small part of the iodine of the thyroxine molecule. Comparing the radioactivity of the blood from the foetuses which received thyroxine injection with that of the mother animals, it can be stated that only negligible radioactivity was found in the latter. The radioactivity of the blood and amniotic fluid samples of the intact foetuses was generally similar to that of the maternal blood. In the amniotic fluid samples of one experiment, a discrepancy due perhaps to technical difficulties can be found. The dilution

TABLE 2

RADIOACTIVITY OF VARIOUS SAMPLES AFTER INJECTION OF RADIOACTIVE THYROXINE INTO ONE OF THE FOETUSES

Experiment No.	Duration of Test (hr)	Foetus No. <sup>1</sup>	Weight of Foetus (g)	Radioactivity (Counts per Min.) <sup>2</sup>				Weight of Pregnant Guinea Pig (g)	Thyroxine Dose ( $\mu$ c)
				Foetal Samples ( $\frac{1}{4}$ cc)		Maternal Samples ( $\frac{1}{4}$ cc)			
				Blood	Amniotic Fluid	Blood	Bile		
1	3	1	35	12	2	4	4	1000	1
		2	35	9	1				
		3	40	234					
2	$2\frac{2}{3}$	1	30	9	5	9		800	1
		2	40	12	8				
		3	35	209					
3	4	1	35	0	0	0	0	950	3
		2	45	0	0				
		3	35	105					
4	$\frac{1}{2}$	1	110	6	0	5		1000	1
		2		0	8				
		3		0					
		4		238					
5	4	1	150	0	22	0	62	880	3
		2		0	53				
		3		21	69				
6	1	1	80	0	0	7	16	900	3
		2	70	300	412				

<sup>1</sup> The numbers of the foetuses receiving radioactive thyroxine have been printed in bold type.

<sup>2</sup> The value of the background (25 to 31 c. p. m.) has been subtracted from each of the recorded radioactivity values.

conditions discussed in connection with the iodine experiments applied to the thyroxine tests too.

There is a clear difference in the behaviour of iodine and thyroxine. The former no doubt permeates the guinea pig placenta. This is in agreement with previous observations (2, 6). As for the latter, the question is not so clear. However, one has the impression that if thyroxine permeates the placenta, this does not easily take place in the first hours after the administration. Previous

tests with thyroxine injected into the mother animal showed that the guinea pig placenta is practically impermeable to thyroxine similar time intervals (6). Both indirect long-term experiments and clinical observations indicate that thyroid hormone or thyroxine is able to pass through the placenta (4, 5, 9, 10, 11). This applies not only to the placental transfer from mother to foetus but also to passage in the opposite direction. The myxoedematous mother of a normal baby may lose her symptoms during pregnancy; these return after parturition (1).

In the experiments in which the mother guinea pig received the radioactive substance (6) the animal was anaesthetized only at the end of the interval between injection and operation. In the present test «Nembutal» narcosis was already started before the injection. However, this has hardly any essential influence on the results. The radioactive iodine behaved similarly in both experiments too. It was found in the blood on both sides of the placenta. Both the radioactive iodine and the thyroxine test tended to show that the permeability of the placenta is independent of the passage direction.

The various papers on the permeability of the placenta to thyroxine or thyroid hormone are based on observations of different species, such as man (1, 9), dog (3), rabbit (7), rat (4, 5) and guinea pig (5, 6, 10). Not only this, but also the permeability conditions after a somewhat longer time than was used in the present test, require further studies. In addition to this, it is possible that a thyroxine dose larger than those used in the present test may permeate the placenta more easily.

#### SUMMARY

The permeability of guinea pig placenta to radioactive inorganic iodine ( $I^{131}$ ) and thyroxine (labeled with  $I^{131}$ ) was studied. The radioactive compound was injected into one of the foetuses. The experiments with radio-iodine showed a distribution of iodine, not only in the maternal circulation, but also in intact foetuses, indicating a double passage through the placenta. Half an hour to 4 hrs after the injection of 1 to 3 microcuries of radiothyroxine, only very slight radioactivity or none at all was found in the blood of the mother animal and intact foetuses.

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ACTIVITIES OF MICROCOCCI  
ANALYSIS OF 150 STRAINS

I

BIOCHEMICAL REACTIONS

by

M. E. PARMALA

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It may often be difficult to establish the pathogenicity of micrococci *in vitro* since no absolute criterion has been presented as yet on the laboratory diagnosis of pathogenic and non-pathogenic strains. Among the *in vivo* methods, only the rabbit skin test may be taken into consideration, as it usually gives good results (36), but it is seldom in routine use. Among the *in vitro* methods, the coagulase test, the fermentation of mannitol as well as the tests for alpha and beta toxins (haemolysins) are considered to give the best information (4, 36). Furthermore, mainly the phosphatase test (2) and fibrinolysin test (39) have been recommended.

The present work<sup>1</sup> deals with 150 micrococcal strains. The aim was to study their biochemical activity. An effort was also made to find out the suitability of the routine laboratory methods for the establishment of pathogenicity of micrococci.

*Material.* — The strains, with the exception of the Oxford and the 209 P type strains as well as the Norwegian serologic type strains (29), 17 A, 2253, 365 S, 1503, 28, F 21, 2095, 3647 and

<sup>1</sup> The work is part of a larger study dealing with the qualities of micrococci.



3189,<sup>1</sup> have been isolated in the course of one year from samples sent to the Bacteriological Department for routine analysis as well as from nasopharyngeal swabs of healthy people. Forty-four strains from children's diarrheal stools<sup>2</sup> and 14 strains from nasopharyngeal swabs have been isolated with a selective potassium chromate medium containing 2.0 g of  $K_2CrO_4$  in 500 cc. of 10 per cent blood agar (33). The remaining 81 strains were isolated as follows: urine 20, stool of healthy people 6, pyodermial cases 12, pus 6, eczema 26, secretion from the eye 1 or ear 5, sputum 2 and 3 strains of unknown origin.

*Methods.* — The coagulase test was performed according to Fisk (22) with the following modification. The strains were incubated for two days in an atmosphere containing 30 per cent  $CO_2$  at 37°C. Human plasma was diluted with nine parts of sterile saline, and 0.5 ml of broth culture was added and the tubes were incubated at 37°C in a water bath. Observations were made at half-an-hour intervals. After four hours, no more positive results were observed.

The haemolysins were tested according to McFarlan (21) in tubes with red cells from sheep. Observations were made at three-hour intervals. After six hours, no more positive results were observed.

The fermentation of mannitol, lactose, sucrose and salicin was produced in a broth containing 0.5 per cent substance to be tested (23). The readings were taken after 24 and 48 hours.

The fibrinolytic activity was tested on plates poured with a medium containing 10.0 g. of lyophilised fibrinogen<sup>3</sup> in 300 cc. of 2 per cent nutrient agar. The test was repeated on plates containing, correspondingly, 90.0 g. of fresh fibrinogen fraction (which contained some remains of haemoglobin), which was suspended in Waring blender with *quantum satis* of saline to make a smooth suspension. The fibrinogen plates were incubated for 24 and 48 hours at 37°C. The positive result was noted as a clear zone around the colonies.

The coagulase slide-test was performed according to Williams & Harper (45).

<sup>1</sup> Placed kindly by Dr. Oeding at my disposal.

<sup>2</sup> Placed kindly at my disposal by Dr. Rantasalo from the State Serum Institute, Helsinki.

<sup>3</sup> Plasma fraction I (Cohen) made with cold ethanol precipitation from pooled human plasma (kindly placed at my disposal by the State Serum Institute, Helsinki).

The phosphatase micro-test of White & Pickett (44) was modified as follows: 0.05 g of disodium phenylphosphate was dissolved into 100 ml of 0.01 molar McIlvaine phosphate-citric acid puffer (pH 5.6). Of this solution, 0.5 ml was pipeted into sterile tubes and inoculated heavily from agar plate colonies. After four hours' incubation at 37°C in a water bath, 4—5 drops of a 0.5 per cent methanol solution of 2,6-dibromchinonchlorimide (Merck) was added in tubes and allowed to stand for 15 minutes at room temperature. 0.3 ml of n-butanol was mixed with it and allowed to stand for five minutes. The positive result came out in blue colour of the butanol layer.

The pigment formation was produced by means of milk agar plates after 24 hours' incubation at 37°C and after an additional 48 hours' incubation at room temperature.

The liquefaction of gelatin was tested on plates containing 3 per cent gelatin in a 2 per cent nutrient agar. The plates were incubated for 48 hours at 37°C and then a solution containing 15 g of  $\text{HgCl}_2$  and 20.0 g of concentrated HCl in 100.0 ml of  $\text{H}_2\text{O}$  was poured on the plates. The positive result came out as a clear zone around the colony.

Lipolysis was tested according to Starr (43) with some modification. The oil emulsion to the spirit-blue agar was made by mixing 10 ml olive oil in Waring blender with 1 g of fine gum Arabic and 40 ml of warm water. The lipolysis was indicated by a deep greenish-blue colour around the colony.

The reduction of nitrate was tested according to Kauffmann (23) with a mixture containing the acetic acid solutions of sulphanilic acid and alphanaphthylamine.

The methyl-red reaction was tested in a Bacto-Proteose Peptone No. 3 (Difco) broth with glucose and read after four days' incubation at 37°C.

Caseolysis was produced on milk agar plates containing 150 ml of skimmed milk in 1,000 ml of 2 per cent nutrient agar. Incubation was performed at 37°C. The lysis was indicated after 24 and 48 hours by a clear zone around the colonies.

The ability to grow with ammonium as the only source of nitrogen was observed in a liquid medium containing 0.5 per cent ammonium and 0.5 per cent glucose (23). The incubation time was four days at 37°C.

The fermentation of starch was estimated on plates containing 2 per cent water soluble starch in 2 per cent nutrient agar. After 48 hours' incubation at 37°C, the residual starch was demonstrated with an iodine solution.

The catalase was studied by pouring 3 per cent hydrogen peroxide solution on the colonies grown on a 2 per cent nutrient agar and observing the gas formation.

### RESULTS

All strains were catalase positive gram+cocci appearing in pairs or in irregular groups. None of them was able to grow with ammonium as the only source of nitrogen or to ferment the starch. The results of other reactions are given in Table 1.

TABLE 1

THE NUMBER OF POSITIVE RESULTS AND THE PERCENTAGE IN GROUPS OF OBVIOUSLY PATHOGENIC AND NON-PATHOGENIC STRAINS AS WELL AS IN THE WHOLE MATERIAL

	Obviously Pathogenic 114 Strains		Non-pathogenic 36 Strains		Whole Material 150 Strains	
	No.	%	No.	%	No.	%
Coagulase, tube .....	114	100	—	0	114	76.0
Haemolysis .....	92	80.7	—	0	92	61.3
Mannitol fermentation .....	110	96.5	4	11	114	76.0
Fibrinolysis .....	108	94.7	5	14	113	75.3
Coagulase, slide .....	103	90.3	3	8	106	70.7
Phosphatase .....	87	76.3	3	8	90	60.0
Yellowish to orange pigmentation	109	95.6	—	0	109	72.7
Gelatinolysis .....	107	93.8	22	61	129	86.0
Lipolysis .... .	111	97.4	18	50	129	86.0
Lactose fermentation .....	109	95.6	24	66	133	88.7
Sucrose fermentation .....	112	98.3	33	92	145	96.7
Nitrate reduction .....	114	100	28	78	142	94.7
Methyl red .....	111	97.4	31	86	142	94.7
Caseolysis .....	—	0	7	19	7	4.7
Salicin fermentation .....	—	0	2	6	2	1.3

The type strains Oxford and 209 P were alike as to their biochemical reaction except in the formation of haemolysin, which was absent with the latter. The most important reactions and the base of pathogenicity diagnosis are indicated in Table 2.

TABLE 2

THE DISTRIBUTION OF STRAINS INTO GROUPS ON THE BASIS OF THE MOST IMPORTANT BIOCHEMICAL REACTIONS

Coagulase Tube	Haemolysis	Mannitol Fermentation	Fibrinolysis	Coagulase, Slide	Phosphatase	Yellowish to Orange Pigmentation	No. of Strains in Subgroups	Pathogenic	Doubtful Pathogenic	Non- pathogenic	No. of Strains in Groups	
+	+	+	+	+	+	+	63	+				Oxford
+	+	+	+	+	—	+	16	+				
+	+	+	+	+	—	—	1	+				
+	+	+	+	—	+	+	4	+				
+	+	+	+	—	+	—	1	+				
+	+	+	+	—	—	+	1	+				
+	+	+	—	+	+	+	3	+				
+	+	+	—	—	+	—	1	+			90	
+	+	—	+	+	+	+	1	+			1	
+	—	+	+	+	+	+	11	+				209 P
+	—	+	+	+	—	+	5	+				
+	—	+	+	—	+	+	2	+				
+	—	+	+	—	—	+	1	+			19	
+	—	+	—	—	—	—	1		+			
+	+	—	—	—	—	—	1		+			
+	—	—	+	+	+	+	1		+		4	
—	—	+	—	—	+	—	1			+		
—	—	—	+	—	+	—	3			+		
—	—	—	+	—	—	—	1			+		
—	—	—	—	—	—	—	4			+		
—	—	—	—	+	—	—	3			+		
—	—	—	—	—	+	—	1			+		
—	—	—	—	—	—	—	23			+	36	

Of the 36 «non-pathogenic strains», four were mannitol-positive, but like all the others coagulase-negative and not forming haemolysins. Of the «non-pathogenic strains», five were fibrinolytic and four of these simultaneously gelatinolytic and caseolytic i.e., strongly proteolytic. On the other hand, there were three strains which were not fibrinolytic although they were both gelatinolytic and caseolytic. Consequently, all proteinolytic strains cannot be considered fibrinolytic. Using Schubert's (38) division, the «non-pathogenic strains» have been divided as follows:

Micrococcus candidus .....	3
»           »   (mannitol fermenting) ..	1
»           »   methyl red negative....	1
»       albus apathogenic .....	2
»       epidermidis .....	16
»           »   (no gelatinolysis) ....	9
Unidentified micrococcal strains.....	4

Eight of the *M. epidermidis* strains were atypical (2 methyl red negative, 4 caseolytic and 2 lactose and/or sucrose negative) and four of the *M. epidermidis* strains (not gelatinolytic) were atypical (2 methyl red negative and 2 lactose negative). Four unidentified strains which did not fit in Schubert's division were:

*Strain 58*: white pigmented strain, which was gelatinolytic and caseolytic, fermented sucrose but not mannitol and did not reduce nitrates;

*Strain 59*: white pigmented strain, which was gelatinolytic but not caseolytic, fermented sucrose but not mannitol and did not reduce nitrates;

*Strain 63*: white pigmented strain, which was gelatinolytic and caseolytic, fermented sucrose and salicin but not mannitol or lactose, and did not reduce nitrates. However, the methyl red test was positive;

*Strain A 48*: white pigmented strain, which was gelatinolytic and caseolytic, fermented sucrose, salicin, mannitol and lactose, and reduced nitrates. The methyl red test was positive;

The last two strains cannot be placed, according to Bergey's Manual (3), to any of the groups described.

#### DISCUSSION

The determination of coagulase is usually performed only in tubes. Part of the strains may give here a negative result, although the same strain turns coagulase-positive after a few plate passages (25, 30). In addition, the lysis of coagulum caused by fibrinolysis adds to the wrong results (13) as well as to a slow coagulum formation. The R-forms are usually fast-clotting and the S-forms slow-clotting types (41) which makes it necessary to follow the tubes

from 3 to 24 hours. In order to eliminate this defect, plasma plate tests have been developed (31, 34). They do not, however, always give useful results (47), because of the 151 strains, of which 86 were coagulase positive by the tube method, 4 gave wrong negative, and as many as 22 wrong positive results. It is also more difficult to read the plates (13) than the tubes. Better results than the ordinary plasma plates were obtained by plates containing fibrinogen purified by salt precipitation (47).

According to Duthie (15), there are two forms of coagulase, cell-bound and free. The latter activates the fibrinogen directly, the effect of the former being kinase-like and activating prothrombin. In Duthie's opinion, the slide test which shows the free coagulase is an adequate test for pathogenicity. The slide and the tube tests do not, however, give identical results. According to Williams & Harper (45), 88 of the 100 tube positive strains were clearly and 7 doubtfully positive on slides. One of the tube negative strains was doubtfully positive in slide test and the other did not form any clump but the bacteria agglutinated.

This material consisted of 25 (21.9 per cent) strains which, being recently isolated, were coagulase-negative in tubes but after a few plate passages gave positive results. Here we have a phenomenon described by Lominski and coll. (25). The exceptionally high number of these strains is due to the fact that in collecting the strains, the coagulase negative ones were selected from obviously pathogenic processes. In the slide test, 12 wrong negative and 3 wrong positive reactions were obtained. Contrary to Duthie's viewpoint, we think that this test is not sufficiently reliable.

As the pathogenicity is generally established by the coagulase test, the results obtained by Shaw and coll. (40) attract attention. Among the 143 coagulase-positive strains examined by them, 3 per cent did not reduce nitrate, 6 per cent fermented starch and as many as 22 per cent fermented salicin. According to Bergey's Manual (3), none of these characteristics belongs to the type *micrococcus pyogenes*. We may agree with Lack and coll. (24) who doubt that the coagulase test gives too many wrong positive results; in other words, that all coagulase-positive strains are by no means pathogenic. Marks (28) also concurs with this and thinks that only the establishment of alpha haemolysins justifies considering a strain pathogenic.



The mannitol fermentation in aerobic conditions may be among one of the poorest indicators used for the establishment of pathogenicity. Thus in Christie's material (8), 55 (26 per cent) of the 210 clearly non-pathogenic strains were mannitol positive and 6 (0.8 per cent) of the 763 clearly pathogenic strains were negative. Among Evans's (18) 44 non-pathogenic (coagulase-negative) strains 36 (81 per cent) were positive. Also, the *in vivo* experiments made with mice (39) have proved that the mannitol fermentation has no absolute criterion with the virulence. Of the probably pathogenic strains in this material, 5.3 per cent were negative and of the non-pathogenic ones, 11 per cent were positive.

The results of the phosphatase test should correlate nearest to the coagulase. Bray and coll (5) who developed the method, however, found that both the albus and the aureus strains were rich in phosphatase. Also *micrococcus ureae* and *micrococcus sarcina* were phosphatase-positive in his material. Barber and coll. (1, 2) who applied the method to the micrococcal tests obtained a 100 per cent positive result with pathogenic strains and extremely few wrong positive ones among the non-pathogenic strains. Later Gupta and coll. (20) obtained a 59 per cent positive phosphatase with coagulase-negative strains. Carrere and coll. (7) observed that after 24 hours' incubation 23/32 pathogenic strains on the plates and 28/32 pathogenic strains in the tubes were positive but only 1/10 of the nonpathogenic strains. After 48 hours, the number of positive pathogenic strains had amounted to 31 and that of positive non-pathogenic ones to 5. According to these results, phosphatase is encountered frequently also with non-pathogenic strains. This material which is based on the rapid micro method, gave too many positive results only in about 8 per cent of the non-pathogenic strains but as much as 23.7 per cent of the results were negative with the pathogenic strains. This is in disagreement with the results of White and coll. (44), due perhaps to the low number of strains examined by them. It seems that the rapid micro method gives too few positive results whereas the plates give too many, and neither of them can be considered sufficiently accurate to be done alone.

According to current literature, the fibrinolysin determination by micro tests has given rather varying results even with human strains. Madison (26) observed in superficial human processes made in tubes that only 23 per cent were fibrinolytic strains whereas in



deep-seated lesions they amounted to 90 per cent. The results obtained by Christie (10) with human strains on fibrinogen plates were positive in 92 per cent of cases. His results published later in a more extensive material (12) revealed that with clearly pathogenic strains 95 per cent, with doubtfully pathogenic strains 93 per cent and with non-pathogenic strains only 0.5 per cent were positive. According to Lack (24), only 68.5 per cent of the coagulase-positive strains were fibrinolytic. The differences in fibrinogens (27) must have affected differences in the results. In strains isolated from domestic animals, the fibrinolytic strains have been met only exceptionally (26, 27). This may be due either to the inhibiting effect of beta-haemolysin (10) or to the fact that beta-haemolytic strains do not possess this ability (9, 37). However, fibrinolysin is not identical with alpha-haemolysin. These experiments using fibrinogen obtained from pooled human plasma gave a positive result in 108 strains (94.5 per cent) of the coagulase-positive ones and likewise a positive result with 5 strains of the 36 non-pathogenic ones. Of these 5 wrong results, 4 were strongly proteolytic just as was Christie's (10) wrongly positive strain. There was only one (0.7 per cent) really wrong positive non-pathogenic strain isolated from a diarrheal stool. Of the six strains which might have been erroneously negative, three represented the albus type (one isolated from a skin secretion and two from diarrheal stools) and three the aureus type (one isolated from a skin secretion, one from a diarrheal stool and one from external otitis). None of them, consequently, represented a process which would imply an absolutely pathogenic strain.

The haemolysis tests on plates, on which the quality of the toxin is not determined, are not sufficient for the establishment of pathogenicity (16) although the plate test is more sensitive than the tube test (17) in which, if rabbit blood is used, the rabbit may have natural antibodies which disturb the lysis (32). Several non-pathogenic strains also contain haemolysins (16, 35), which do not neutralize with the alpha antitoxin. Maybe it is due only to a proteolytic enzyme activity and to toxic haemolysins (28). It has been claimed that the establishment of alpha toxin in a routine way is no absolute criterion for pathogenicity (16, 42) and that the beta toxins may hinder the lytic effect of the alpha toxin (17, 9). In addition, even in a natural population there are numerous mutants

with regard to different haemolysins (17). Yet many investigators (12, 28, 46) stress that only the establishment of alpha toxins is a reliable indicator for pathogenicity. In plate passages the toxin formation may disappear (17) as may have happened to the greatest part of type strains in this material. This disadvantage is not, however, encountered when testing recently isolated strains.

Also the lipase formation has been considered to have some correlation with haemolysins and with the pathogenicity. According to many investigators (11, 14, 19), this kind of correlation cannot, however, be established, although the lipolytic strains are in general more active biochemically. This material seems to prove that this opinion is justified. The egg yolk opacity test presented by Gillespie (19) which may show only the lipolytic effect on triglycerids is, however, positive only with an exceptionally small part of the strains and may require additional studies as to its validity as an indicator of pathogenicity.

Consequently, the pigmentation and gelatinolysis do not give even sufficient indications for the establishment of pathogenicity. The coagulase test is, nevertheless, by no means an ideal one, since it gives too many wrong results in tests made soon after isolation required by the clinics. The determination of alpha haemolysins from recently isolated strains on sheep blood agar plates together with antitoxin control may give good results although a part of obviously pathogenic strains may then be ignored. In laboratories testing human strains only, plates made of fibrinogen fractionated from pooled human plasma may give satisfactory results, since alpha-beta combinations with strong beta-toxic effect are rare in human processes (16, 10), and the pure beta-toxic strains are lacking (6). By combining both haemolytic and fibrinolytic determinations a satisfactorily reliable picture may be obtained of the pathogenicity of micrococci.

The grouping of apathogenic strains is still unsettled. Shaw's (40) grouping is far too rough and Schubert's (38) grouping leaves several strains outside the groups even in this small a material. For the present, it might be most practical to keep them in a single group labelled *Micrococcus saprophyticus*.

## SUMMARY

Fifteen biochemical reactions of 150 micrococcal strains were tested. On the basis of the experiment and on literature the values of the different reactions used in the differentiation of pathogenicity were discussed. Fibrinolysis based on plate tests made with fibrinogen obtained with cold ethanol precipitation from pooled human plasma was suggested, with or without alpha toxin determinations, to be the most valuable test in laboratories dealing only with human micrococcal strains.

The classification of non-pathogenic micrococci seems not be solved as yet. A single name *Micrococcus saprophyticus* was suggested to be used commonly with all the non-pathogenic micrococcal strains.

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## ACTIVITIES OF MICROCOCCI

### ANALYSIS OF 150 STRAINS

#### II

#### ANTIBIOTIC SENSITIVITY

by

M. E. PARMALA

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Many reports of the antibiotic sensitivity of micrococci and the changing pattern of their sensitivity have been published in the last few years. However, we have seen only one paper dealing with fecal micrococci strains in the literature (2). In that paper are 14 strains described, almost all isolated from patients who had got antibiotics previously.

The strains studied in the present work are the same as in a previous paper (1). The sensibility tests was made with disc technique using Bacto sensitivity discs. Antibiotics tested were: penicillin, dihydrostreptomycin, chlortetracyclin, oxitetracyclin, tetracyclin, chloramphenicol, erythromycin, carbomycin, neomycin, bacitracin and polymyxin B. In addition, gantrisin (3.4-dimethyl-5-sulphanilamido-isoxazole) discs were included.

Results and antibiotic concentrations in discs are given in tables 1—4.

It seems, that the fecal strains are significantly more resistant to penicillin, chlortetracyclin, oxitetracyclin, tetracyclin and carbomycin than the other non-fecal strains of micrococci. Among fecal strains there are also remarkably few sensitive strains against erythromycin, neomycin and bacitracin. Since the fecal strains are

TABLE 1  
SENSITIVITY OF 44 COAGULASE POSITIVE FECAL STRAINS (PER CENT)

Antibiotic	Concentration	—	—?	±	Partly	+
Penicillin .....	1 unit	82	7	9	—	2
Dihydrostreptomycin ....	10 mcg	34	—	14	5	48
Chlortetracyclin .....	30 "	30	7	32	—	32
Oxitetracyclin .....	30 "	43	2	36	2	16
Tetracyclin .....	30 "	45	2	27	—	25
Chloramphenicol .....	30 "	—	—	2	2	95
Erythromycin .....	1 "	—	—	36	—	63
Carbomycin .....	1 "	9	—	86	5	—
Neomycin .....	30 "	—	—	50	7	43
Bacitracin .....	10 units	—	2	70	—	27
Polymyxin B .....	10 mcg	50	50	—	—	—
Gantrisin <sup>(R)</sup> .....	50 "	75	7	5	14	—

— = no zone    —? = 1 mm zone    ± = 2–4 mm zone  
partly = some resistant colonies in zone    + = zone 5 mm or more

TABLE 2  
SENSITIVITY OF 70 COAGULASE POSITIVE NON-FECAL STRAINS (PER CENT)

Antibiotic	—	—?	±	Partly	+
Penicillin .....	51.4	4.3	11.4	7.1	25.7
Dihydrostreptomycin	30	1.4	5.7	1.4	61.4
Chlortetracyclin .....	14.3	1.4	48.5	4.3	31.4
Oxitetracyclin .....	18.5	—	35.7	4.3	41.4
Tetracyclin .....	22.8	—	48.5	2.8	25.7
Chloramphenicol ....	1.4	—	8.5	2.8	87.1
Erythromycin .....	7.1	—	—	—	92.8
Carbomycin .....	1.4	—	17.1	4.3	77.1
Neomycin .....	2.8	—	21.4	1.4	74.3
Bacitracin .....	2.8	—	37.1	5.7	54.3
Polymyxin B .....	32.8	25.7	40.0	1.4	—
Gantrisin <sup>(R)</sup> .....	75.7	1.4	1.4	18.5	2.8

collected from babies suffering from diarrhoe (39 aureus and 5 albus strains) as well as from healthy adults (5 aureus and 1 albus strain) there is some evidence that the relative resistance of the isolated fecal strains is not due to an antibiotic therapy but that they are perhaps naturally more resistant to the antibiotics enlisted, even if the fecal strains do not differ in any other biochemical reaction from their strains.

TABLE 3  
SENSITIVITY OF 6 COAGULASE NEGATIVE FECAL STRAINS (NO)

Antibiotic	—	—?	±	Partly	+
Penicillin .....	2	1	2	—	1
Dihydrostreptomycin	2	—	—	—	4
Chlortetracyclin .....	—	2	3	—	1
Oxitetracyclin .....	2	1	3	—	—
Tetracyclin .....	3	—	3	—	—
Chloramphenicol ....	—	—	—	—	6
Erythromycin .....	—	—	—	—	6
Carbomycin .....	—	—	6	—	—
Neomycin .....	—	—	1	—	5
Bacitracin .....	—	1	4	—	1
Polymyxin B .....	2	3	1	—	—
Gantrisin <sup>(R)</sup> .....	3	—	—	1	2

TABLE 4  
SENSITIVITY OF 30 COAGULASE NEGATIVE NON-FECAL STRAINS (PER CENT)

Antibiotic	—	—?	±	Partly	+
Penicillin .....	17	13	30	—	40
Dihydrostreptomycin	13	—	7	—	80
Chlortetracyclin .....	17	—	33	—	50
Oxitetracyclin .....	13	—	40	—	47
Tetracyclin .....	17	—	50	—	33
Chloramphenicol ....	3	—	—	—	97
Erythromycin .....	—	—	3	—	97
Carbomycin .....	7	—	57	—	36
Neomycin .....	—	—	3	3	94
Bacitracin .....	—	—	67	—	33
Polymyxin B .....	7	7	86	—	—
Gantrisin <sup>(R)</sup> .....	57	—	—	20	23

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## STAPHYLOKINASE AND STAPHYLOCOAGULASE

### A COMPARATIVE STUDY

by

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In a previous work (9) one of us stated that fibrinolysin formation on plates containing fibrinogen fractionated from pooled human plasma would be helpful in laboratory diagnosis of pathogenicity of micrococcal strains. Since the material was too small to give an accurate picture of the value of fibrinolysis test, a larger comparative serie was performed.

*Material.* — This consisted of 700 micrococcal strains of human origin. Of the strains 654 were isolated from various clinical samples and 46 strains from diarrhoeal stool of babies.<sup>1</sup>

*Methods.* — Coagulase tests were made with two methods. All our control determinations, as well as 60 per cent of the primary routine determinations, were made from plate cultures in tubes containing 1 ml 1:10 saline dilution of human plasma, read after 20 hours' incubation at 37°. In the remaining 40 per cent of primary determinations a loopful of plate culture was mixed with broth and 0.8 ml of it was mixed with 0.2 ml of human plasma. The result was read after 2 hours' incubation at 37° in a water bath.

The staphylokinase or fibrinolysis tests were made with plates containing fibrinogen fractionated with cold ethanol precipitation (1) from pooled human plasma. The fibrinogen »paste» was kept in

<sup>1</sup> The latter strains as well as 234 of the former strains were kindly set at our disposal by the State Serum Institute, Helsinki.

a deep freezer until used. From the stock, 50 g of the «paste» was mixed in Waring Blendor with 50 ml of sodium-citrate buffer solution (pH 5.9) (5) kept at 4°. Immediately after the «paste» was thoroughly mixed with buffer avoiding foaming it was gently stirred with 500 ml of 2 per cent nutrient agar at 56°, and the plates were poured. During the solidification the plates become turbid. Kept in refrigerator at +4°—+8° the plates were useful about one weeks time. (Too old plates will clear up by themselves).

A loopful of bacteria from original agar plate was inoculated as a spot, 12 to 15 strains in every plate. Incubation was performed at 37°, and the reading was done after 18—20 hours, but in later series the plates were kept at room temperature 24 hours only with conforming results. At room temperature there was a lesser tendency to self clearing of old plates. The result was considered positive if a clear zone of about 2—3 millimeters or more was seen around the colony. Negative strains did not split the fibrinogen at all.

Results of the comparative tests are given in table 1.

TABLE 1

COAGULASE AND STAPHYLOKINASE ACTIVITY OF 700 MICROCOCCAL STRAINS

	Coagulase	Kinase	Number	Per Cent
Agreeing strains .....	+	+	442*	63.14
	—	—	168	24.00
	Total		610	87.14
Disagreeing strains .....	+	—	46	6.57
	—	+	44	6.29
	Total		90	12.86

\* 30 (6.8 per cent) of the 442 coagulase and kinase positive strains were coagulase negative when isolated, but became later positive.

The lysis of fibrinogen in plates occurred within a few hours at 37° or after an overnight incubation at room temperature.

Some additional experiments were performed to study the nature of fibrinolytic activity. Positive micrococci, beta-hemolytic streptococci and saline dilutions of streptokinase<sup>1</sup> were dropped on to a plate and incubated at 37°. In one and a half hours' time there was a clear liquefaction zone around drops of 10,000 units and 1,000

<sup>1</sup> Varidase Lederle, kindly set at our disposal by Messrs Drugtrade Oy Ab, Helsinki.

units of streptokinase and also around the micrococcal colony, streptococci colony being not yet visible. After four and a half hours' incubation the zone around 1,000 units of streptokinase was 8.5 mm, that around micrococcal colonies 5 mm and that around streptococcal colonies 2 mm. Turbidity around and under plain saline drops was unaltered.

A clot of original fibrinogen »paste» and fibrin made of it by saline dissolution and clotting with thrombin solution were both entirely dissolved by streptokinase within ten minutes at 37° in a water bath.

#### DISCUSSION

As Lominski and others (4, 7, 9) have stated, some recently isolated micrococcal strains do not coagulate plasma, but will do it later after a few plate passages. All coagulase positive strains are also by no means always pathogenic (3, 9). Therefore it would be justifiable to get some other rapid tests for laboratory diagnosis of pathogenicity of micrococci. Much (6) was the first to observe fibrinolytic activity of micrococci; later, the object was more closely studied by many investigators (cf. 9).

The present material gives a good evidence of the suitability of the fibrinogen plates to the pathogenicity tests. As they contain only one fraction made from pooled human plasma, they do not contain any antibodies, which may disturb the usual coagulase test making some plasmas unsuitable for the coagulase determinations.

Lack (2) stated that the action of micrococci to fibrin is due to their kinase activity upon plasminogen and not to any specific proteolytic enzyme. Cohn and coll. (1) have shown that the first ethanol fraction of plasma contains some plasminogen, prothrombin and gamma-globulin. Oncley and coll. (8), however, stated that little or no plasminogen is to be found in any other fraction than  $\text{III}_2$  and  $\text{III}_3$ . But as the original fibrinogen »paste» and fibrin made from it are both rapidly dissolved by 1,000 units of streptokinase per ml, our fraction must contain enough of plasminogen. Accordingly we incline to accept the view that the micrococcal activity is with most probability due to kinase action.

## SUMMARY

700 micrococcal strains were tested for coagulase and staphylokinase activity. There was a good conformity with both tests. When the coagulase test gave transitory negative results in 6.8 per cent of recently isolated strains, the staphylokinase test (fibrinolysin test) was constantly positive with positive strains. The fibrinogen fraction used did enough plasminogen.

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## PHENOLPHTHALEIN PHOSPHATE-CHROMATE PLATE

### A SELECTIVE MEDIUM FOR ISOLATING AND DIFFERENTIATING MICROCOCCAL STRAINS

by

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It is often difficult to establish the micrococci in samples rich of gram-negative rods, especially proteus species. Therefore it would be desirable to get a medium which is strongly selective and gives some evidence of the pathogenicity of the isolated micrococcal strains. As Rantasalo (5) has observed, the isolation of pure cultures of micrococci from stool specimens is possible with blood plates containing potassium chromate or bichromate (2). Phenolphthalein phosphate plates have according to some investigators (1,5) had some correlation with the pathogenicity.

The material consisted of 550 micrococcal strains isolated from various clinical specimens.<sup>1</sup>

The coagulase and staphylokinase tests are made as in a previous paper (4). Plates containing phenolphthalein phosphate and potassium chromate were made as follows: To 500 ml of 2 per cent nutrient agar at 56° was mixed 2 gr of potassium chromate ( $K_2CrO_4$ ) dissolved in 10 ml of sterile saline; 0.25 gr of colaminephenolphthalein phosphate (Merck;  $C_{28}H_{44}O_{14}N_4P_2$ ) (3) dissolved in 20 ml of sterile saline, and 50 ml skimmed milk sterilized 20 minutes at 120° in an autoclave (also to destroy the phosphatase enzyme of milk).

<sup>1</sup> Part of the strains were kindly put at our disposal by the State Serum Institute, Helsinki.

After mixing the plates are poured. Ready solidified plates are yellowish turbid.

Inoculation was made from original isolation plates with a platin loop as zigzag lines, about 8 strains in a plate. After 20 hours incubation at 37°  $\text{NH}_4\text{OH}$  solution was dropped on the colonies. Phosphatase positive strains gave an immediate red coloured zone around the colonies, while negative ones remained unaltered. All the strains tested grow well on the plate.

Results of the various tests are given in table.

TABLE 1

COMPARATIVE RESULTS OF COAGULASE, STAPHYLOKINASE AND PHOSPHATASE TESTS OF 550 STRAINS

	Coagulase	Kinase	Phos- phatase	No.	Per Cent of Strains
Agreeing results ....	+	+	+	386	70.2
	—	—	—	84	15.3
	Total			470	85.5
»Possible false«					
Coagulase results ..	—	+	+	16	2.9
	+	—	—	15	2.7
	Total			31	5.6
»Possible false«					
Kinase results .....	+	—	+	30	5.5
»Obviously false«					
Phosphatase results	+	+	—	5	0.9
	—	—	+	14	2.5
	Total			19	3.4

Negative coagulase tests are controlled after some plate passages and the correct results are given in table.

It seems that the phenolphthalein phosphate-chromate medium gives quite an accurate indication of the pathogenicity of micrococcal strains compared with coagulase and staphylokinase tests. When the chromate concentration of plates have proved to be selective to micrococci (5), the plate may be helpful in isolation and differentiation of micrococci from specimens containing mixed microbial flora. Further studies of its use in primary isolation media are strated.

## SUMMARY

A selective and differentiating medium containing colamine phenolphthalein phosphate and potassium chromate is described. Tested with 550 micrococcal strains it gave a good evidence of their pathogenicity since only 3.4 per cent of the strains gave false positive or negative result compared with coagulase and staphylokinase tests.

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## Le<sup>b</sup> ANTIGEN

STUDIES ON ITS OCCURRENCE IN RED CELLS, PLASMA AND SALIVA

by

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Serological research has not been able to bring nearly enough order into the Lewis blood group system. When the Lewis groups were discovered [Mourant 1946 (7) and Andresen 1948 (1, 2)], they were included into one and the same system since Le<sup>a</sup> and Le<sup>b</sup> antigens occurred interdependently in the blood of adults. Although it has been observed later that this interdependence is not due to an allelomorphic pair of genes, these blood groups have usually been dealt with together.

The system seems to differ essentially from other blood group systems. The Le<sup>b</sup> antigen is weaker in the red cells of A<sub>1</sub> and A<sub>1</sub>B persons than in those of O, B, A<sub>2</sub> and A<sub>2</sub>B persons (2). The Le<sup>a</sup> gene seems to be dominant in infants and to become recessive by and by during the 1st year of life (1, 2). Cord blood, on the other hand, usually belongs to the Le(a—b—) group like the blood of fetuses in so far as it has been examined (8). Lewis characteristics have been found to be separable from cells by washing (6); moreover, J. and P. Sneath have recently shown that the Lewis groups of cells can be changed into another corresponding to the foreign plasma in which they are treated (9). Accordingly, the Lewis groups would be characteristics of plasma and tissue fluids rather than actual blood groups. Grubb in 1950 came to a similar conclusion on

different grounds: The Lewis blood group system would seem to be essentially «a serological system of water-soluble mucoids» (4).

Thus it is apparent that the Lewis characteristics of red cells passively reflect the properties of the surrounding plasma. It has been found that plasma and boiled saliva contain Lewis substances that inhibit anti-Le sera (6, 3). Saliva is considerably richer in these substances than plasma.

Two questions have arisen in consequence of the facts stated above.

a) Is it possible to attach the Lewis substances of saliva to red cells? In default of a good anti-Le<sup>a</sup> serum we were only able to study the matter with regard to Le<sup>b</sup> substance.

b) Is the weakness of the Le<sup>b</sup> antigen in A<sub>1</sub> and A<sub>1</sub>B persons and in infants due to the fact that their plasma is poor in Le<sup>b</sup> substance; or is it due to the reluctance of the cells to receive it?

#### MATERIAL, METHODS AND RESULTS

We had a strong anti-Le<sup>b</sup> serum from an A<sub>1</sub>B donor at our disposal.<sup>1</sup> We wish to express our thanks to Dr. A. E. Mourant, who kindly checked the serum and confirmed its anti-Le<sup>b</sup> character.

We used the Sneath technique (9) shaking the red cells in various media (plasma or saliva) at 37° for up to 5 days and testing them every day with anti-Le<sup>b</sup> serum. The medium was always renewed at the same time. The same combination of cells and medium was always set up for two or more times and it always gave the same results.

If the mixture became infected the cells could not be used, of course. Also the ageing of the cells influenced their reactions, old cells reacting more weakly as is seen from table 1.

TABLE 1

THE LE(b—) CELLS WERE TREATED FOR 5 DAYS IN LE(b—) AND LE(b+) PLASMA, RESPECTIVELY, AND TESTED EVERY DAY WITH ANTI-LE<sup>b</sup> SERUM

Plasma	1st day; experiment begun	2nd day	3rd day	4th day	5th day	6th day
Le(b—)	—	—	—	—	—	—
Le(b+)	—	—	(+)	++	+	—?

<sup>1</sup> It also contained some anti-O, which was successfully absorbed with OLe(b—) cells.

When the cells were to be treated in saliva, we found that neither undiluted saliva nor saliva diluted in saline could be used, because the cells were destroyed in a couple of days so that they became unsuitable for the agglutination test. Nor could the cells be treated in pure saline, which destroyed them in the same manner. So we used a mixture that contained plasma of the donor of the cells and different saliva specimens in the proportion of 3 to 1. Because the Le<sup>b</sup> positive saliva used inhibited anti-Le<sup>b</sup> serum at the dilution of 1 to 256, at least, the mixture contained quite appreciable amounts of Le<sup>b</sup> substance.

The cells of an O Le(b—) person were shaken together with the saliva dilutions of O Le(b+) persons. An identical dilution of O Le<sup>b</sup> negative saliva served for a control. In neither case could we make Le(b—) cells positive even once, though the treatment was continued for 5 days. The cells that had been treated with Le(b+) plasma became strongly Le<sup>b</sup> positive already in the course of 2 days. (Table 3).

Yet the saliva as well as the mixture of saliva and plasma we used turned out to be rich in Le<sup>b</sup> blood group substance judging by their ability to inhibit agglutination. The plasma, on the other hand, is markedly poor in inhibiting blood group substances. As a rule the plasma of Le(a+) persons inhibits anti-Le<sup>a</sup> serum in the dilution of 1 to 2 according to Brendemoen (3); we have not found corresponding information on Le<sup>b</sup>. As a result from our own experiments on the relative inhibiting ability of various samples of plasma we found that in plasma milieu agglutination was somewhat stronger than in saline. We also noticed slight differences between different specimens (3 tubes at most when titrating the anti-Le<sup>b</sup> serum in saline in powers of 2 and adding to each tube one drop of the plasma to be examined); these differences, however, did not stand in any relation to the Lewis group of the donor. Agglutination was not inhibited by boiled plasma either. (Table 2).

As to our question b), it is to be remembered that the group interesting us consists of such Le(b—) persons who are secretors of ABH (and Le<sup>b</sup>) substances. According to Grubb's series of 1000 persons (5) there are about 50/420 or 12 per cent of such persons in the blood groups O and A<sub>2</sub> but 70 per cent in the groups A<sub>1</sub> and A<sub>1</sub>B. In this latter group we must include those persons corresponding to the 12 per cent in the groups O+A<sub>2</sub> making about

TABLE 2

ANTI- $\text{Le}^b$  SERUM WAS TITRATED AGAINST O  $\text{Le}(b+)$  CELLS IN SALINE. TO EACH TUBE WAS ADDED ONE DROP OF SALINE, PLASMA OR DILUTED SALIVA AND ALLOWED TO STAND FOR 10 MINUTES BEFORE ADDING THE CELL SUSPENSION

Medium	Anti- $\text{Le}^b$ serum in dilutions of						
	1/1	1/2	1/4	1/8	1/16	1/32	1/64
Saline .....	++	++	++	(+)	—	—	—
Plasma No. 1 (of the donor of the cells) ..... $\text{Le}(b+)$	++	++	++	++	++	(+)	—?
Plasma No. 2 ..... $\text{Le}(b+)$	++	++	++	++	+	(+)	—
Plasma No. 3 ..... $\text{Le}(b+)$	++	++	++	++	++	++	+
Plasma No. 4 ..... $\text{Le}(b+)$	++	++	++	++	++	(+)	—
Plasma No. 5 ..... $\text{Le}(b-)$	++	++	++	++	+	(+)	—?
Plasma No. 6 ..... $\text{Le}(b-)$	++	++	++	++	—?	—?	—
Plasma No. 7 ..... $\text{Le}(b-)$	++	++	++	++	++	(+)	—?
Plasma No. 8 ..... $\text{Le}(b-)$	++	++	++	++	++	++	(+)
Plasma No. 9 (cord blood) $\text{Le}(b-)$	++	++	++	++	++	(+)	—
Plasma No. 10 (cord blood) $\text{Le}(b-)$	++	++	++	++	++	+	—?
Boiled plasma ..... $\text{Le}(b+)$	++	++	++	(+)	—	—	—
Boiled plasma ..... $\text{Le}(b-)$	++	++	++	+	—	—	—
Saliva diluted 1/64 ..... $\text{Le}(b+)$	—	—	—	—	—	—	—
Saliva diluted 1/4 ..... $\text{Le}(b-)$	++	++	++	—	—	—	—

20% of them. The remaining 80% of the group  $A_1$  (and  $A_1B$ )  $\text{Le}(b-)$  secretor of ABH substances consist of those exhibiting a so called suppressive action of  $A_1$  on the  $\text{Le}^b$  characteristic in the erythrocytes. Now the question is, whether this suppression extends to the plasma, which then should be poor in  $\text{Le}^b$  substance or whether it concerns the erythrocytes only, making them reluctant to receive the  $\text{Le}^b$  substance.

Using the same technique as before, we shook the red cells of the group  $A_1$   $\text{Le}(b-)$ , secretor of  $\text{Le}^b$  substance, in the plasma of one of the group  $A_2$   $\text{Le}(b+)$ ; likewise we shook the cells of an O  $\text{Le}(b-)$  person in the plasma of the first  $A_1$   $\text{Le}(b-)$  person. Five different  $A_1$   $\text{Le}(b-)$  secretor blood samples were used. It was found that the  $A_1$   $\text{Le}(b-)$  cells received the  $\text{Le}^b$  characteristic from the positive plasma readily and became  $A_1$   $\text{Le}(b+)$ . Their plasma, on the other hand, did not make O  $\text{Le}^b$  negative cells positive. (Table 3).

TABLE 3

THE DIFFERENT CELLS WERE TREATED IN THE VARIOUS PLASMA, OR SALIVA, SPECIMENS, AND TESTED WITH ANTI-LE<sup>b</sup> SERUM

	Cells tested with anti-Le <sup>b</sup> serum		
	O Le(b—)	A <sub>1</sub> Le(b—) secretor	cord blood O Le(b—) secretor
Plasma of an O Le(b—) person	—		—
Plasma of an O Le(b+) person	++		++
Plasma of an A <sub>2</sub> Le(b+) person	++	++	++
Plasma of an A <sub>1</sub> Le(b—), secretor person .....	—	—	—
Plasma of cord blood O Le(b—)	—		—
Saliva of an O Le(b—) person	—		
Saliva of an O Le(b+) person	—		

Of the cord bloods, on the other hand, all the 20 samples we examined were Le (b—). We carried out similar tests as before with these. Cells of 3 and plasma of 7 donors were used so as to secure the presence of a future Le (b+) person. These cells also became strongly Le<sup>b</sup> positive with ease, whereas the plasma did not alter Le(b—) cells. (Table 3).

## DISCUSSION

The Lewis substances of saliva and plasma are thus seen to react in various ways: in saliva they inhibit anti-Le<sup>b</sup> serum very strongly while not becoming attached to cells; in plasma they do not inhibit, or inhibit weakly, while becoming readily attached to cells. A qualitative difference must accordingly exist. In this connection we feel tempted to refer to Grubb, who reports that also A and O—H substances of saliva and cells seem to show a qualitative difference (4).

Our findings seem to corroborate the view that red cells are but passive carriers and detectors of the Lewis substances of plasma.

## SUMMARY

A study was made concerning the occurrence of Le<sup>b</sup> substance in cells, plasma and saliva. The Le<sup>b</sup> substance of plasma was found not to inhibit the agglutination of the Le(b+) red cells by anti-Le<sup>b</sup> serum; moreover, it was found to attach itself readily

to Le(b—) cells thus making them positive. The Le<sup>b</sup> of saliva proved a powerful inhibitor while not altering the Le group of cells. The Le<sup>b</sup> negativity of persons belonging to the group A<sub>1</sub> Le(b—), secretor of Le<sup>b</sup> substance, as well as that of infants seems to be due to their plasma's lacking in Le<sup>b</sup> substance; in positive plasma their cells become Le<sup>b</sup> positive quite readily.

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EFFECT OF DISULFIRAM (TETRAETHYLTHIURAM  
DISULPHIDE) ON THE ELIMINATION RATE  
OF METHANOL<sup>1</sup>

STUDIES WITH RABBITS

by

MARTTI KOIVUSALO

(Received for publication February 23, 1956)

Hald, Jacobsen and Larsen (9) were the first to describe, in 1948, the sensitizing effect of disulfiram (tetraethylthiuram disulphide, antabuse) on ethanol in human subjects. This effect has been explained by the increased concentration of acetaldehyde in the blood which has been observed after the administration of ethanol to patients or various experimental animals previously treated with disulfiram (2, 8, 12).

There are very few observations on the effect of disulfiram on the metabolism of methanol. According to Gilger, Potts and Johnson (6) there is a two-fold increase in the toxicity of methanol to mice after the administration of disulfiram. Owe-Larsson and Skog (15) demonstrated that disulfiram increases the toxicity of formaldehyde to rats and mice. Formaldehyde is nowadays generally regarded as the intermediate in the metabolism of methanol which is responsible for its toxicity. This paper will present some observations on the elimination rate of methanol from the blood of rabbits previously given disulfiram.

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<sup>1</sup> This investigation has been supported by a generous grant from the Foundation for the Research of Alcohol Problems.



## METHODS

Rabbits weighing 2.5–3.5 kg were used in the experiments. The diet during the experiments was oats and water.

A 15 per cent suspension of crystalline disulfiram in water stabilized with a drop of Tween 80 was given to the rabbits by stomach tube in doses of 0.7 g per kg and 1.0 g per kg. One hour later 0.8 to 1.6 g of methanol per kg was given by stomach tube as a 20 per cent aqueous solution or intravenously in 10 ml of physiological saline. Only methanol or disulfiram was given to the control animals. Blood samples were taken from the ear veins before the beginning of the experiment and then at one to eight hours' intervals.

The blood samples were analyzed for their formaldehyde and methanol contents with the aid of the chromotropic acid reaction modified after MacFadyen (13) and Agner and Belfrage (1).

## RESULTS

Typical examples of blood methanol elimination curves selected from groups of similar curves are presented in figures 1 and 2.

In the experiments recorded in fig. 1 methanol was given per os by stomach tube. A series of rabbits were given 0.7 g per kg disulfiram by stomach tube one hour before the administration of methanol. It is seen from the curves that following a dose of 0.8 g per kg the elimination of methanol in the control rabbits occurred at a constant rate after the resorption was complete and the diffusion equilibrium was reached. The rate of elimination after 0.8 g of methanol per kg was  $0.00040 \pm 0.00008$  mg per kg per minute ( $n = 10$ ). After 1.6 g of methanol per kg the elimination rate was more rapid during the first 10–15 hours, when the blood methanol concentration was high, and the elimination rates for the first five hours after equilibrium varied from 0.0020 to 0.0007 mg per kg per minute in four experiments (average 0.0010). After the blood methanol concentration reached about 1000  $\mu$ g per ml the elimination rate was constant and at the same level as after 0.8 g of methanol per kg.

When disulfiram was previously given to the rabbits the elimination of methanol from the blood was considerably retarded. After 0.7 g disulfiram and 0.8 g of methanol per kg was given per os there still was detectable methanol in the blood after 100 hours, although normally the blood was free from methanol in about 35–45 hours after administration of this amount of methanol.

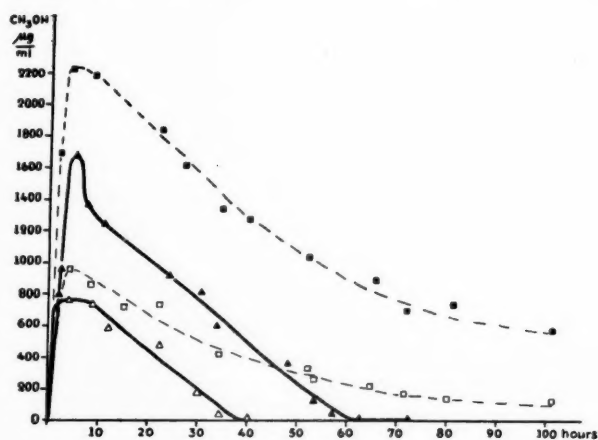


Fig. 1. — Effect of disulfiram on the elimination rate from blood of methanol administered per os.

- △—△ 0.8 g methanol per kg  
 ▲—▲ 1.6 g methanol per kg  
 □---□ 0.7 g disulfiram per kg followed 1 hour later by 0.8 g methanol per kg  
 ■---■ 0.7 g disulfiram per kg followed 1 hour later by 1.6 g methanol per kg

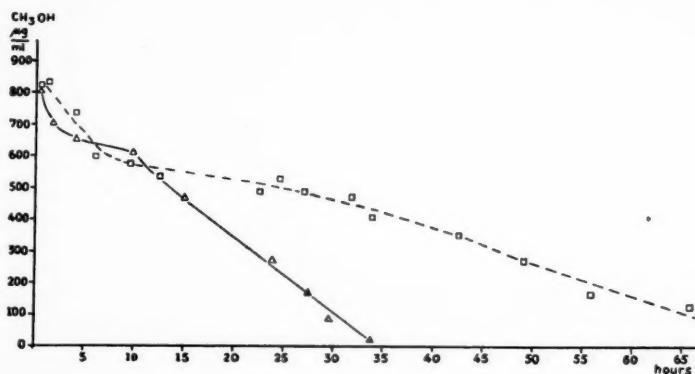


Fig. 2. — Effect of disulfiram on the elimination rate from blood of intra-venously administered methanol.

- △—△ 0.8 g methanol per kg  
 □---□ 1.0 g disulfiram per kg followed 1 hour later by 0.8 g methanol per kg

After disulfiram the elimination rate was very small especially when the blood methanol concentration was low.

The effect of disulfiram is very clearly seen in the experiments where methanol was given intravenously (fig. 2).

During the first ten hours the elimination rate of methanol from the blood in disulfiram-treated rabbits was approximately the same as without disulfiram. After this time it was very low, and was very near zero in many animals during some 10–20 hours. It then gradually increased but nevertheless remained low as compared to the control group. According to Hald *et al.* (10) the maximum effect of a single disulfiram dose on ethanol metabolism begins 12–18 hours after the administration of disulfiram and the effect is very small during the first five hours. In the present experiments disulfiram was given one hour before methanol, so that the time interval before disulfiram reached its maximum effect on the metabolism of methanol was apparently the same as in the case of ethanol.

No formaldehyde was detected in the blood samples of rabbits given methanol or disulfiram and methanol. Disulfiram alone had no effect on the blood formaldehyde or methanol determinations.

The animals had no gross toxic symptoms and recovered fully from the experiments, with the exception of one animal (No. 16, given 0.7 g disulfiram per kg and 0.8 g methanol per kg per os) which was found dead in the morning 30 hours after the administration of methanol. The blood methanol concentration was then 560  $\mu$ g per ml.

#### DISCUSSION

The normal methanol elimination rate from the blood in rabbits according to Bildsten and Widmark (4) is 0.0060 mg per ml per minute and is not correlated to the concentration of methanol in the blood. These values were determined by Bildsten's modification (3) of Widmark's method for the determination of ethanol. According to Agner and Belfrage (1) who used the more specific chromotropic acid method, the elimination rate is 0.0017 mg per ml per minute at the higher methanol blood concentrations and 0.0006 at concentrations below 1000  $\mu$ g per ml. In the present experiments the elimination rate also was constantly slower in the

lower blood concentrations and more rapid when the blood concentration was high. However, at the lower blood methanol concentrations the rate was constantly lower than the value of Agner and Belfrage, being 0.00040 mg per ml per minute.

Hald *et al.* (10) showed that when the amount of disulfiram absorbed exceeds one gram the rate of ethanol elimination is below normal in rabbits and may be as low as zero. They also demonstrated that increasing doses up to one gram of absorbed disulfiram result in increasing amounts of acetaldehyde in the blood when the ethanol concentration is held constant. When more disulfiram is absorbed it has no further effect on the blood acetaldehyde level. The disulfiram concentrations used in the present study were well beyond this saturation limit, and the effect of disulfiram also was clear in all the experiments.

It has been assumed that methanol is metabolized by the same enzymes as ethanol (19). However, the crystalline horse liver alcohol dehydrogenase of Theorell and Bonnichsen (18) had no effect on methanol. The influence of disulfiram on the methanol elimination rate is quite similar to its effect on the elimination of ethanol, although the retarding effect in both cases may be due to general metabolic inhibition after large doses of disulfiram. According to Kjeldgaard (11) the liver alcohol dehydrogenase is not sensitive to disulfiram; however, the disulfiram concentrations used by him were very small.

The action of disulfiram on the metabolism of ethanol has been explained by its inhibitory effect on the enzymes concerned with the metabolism of aldehydes. Kjeldgaard (11) found that disulfiram inhibits the action of the liver flavoprotein aldehyde dehydrogenase already in very low concentrations. Also liver xanthine oxidase (16), d-glyceraldehyde-3-phosphate dehydrogenase (14) and the diphosphopyridine nucleotide-dependent aldehyde dehydrogenase of liver (7) are inhibited by disulfiram. The inhibitory action of disulfiram, however, is not limited to these enzymes but it also causes a more marked inhibition of the endogenous respiration of rat liver homogenates (5, 16) than should be due to inhibition of only the aforementioned enzymes.

If the effect of disulfiram on the metabolism of methanol were analogous to the effect on the metabolism of ethanol there should occur an accumulation of formaldehyde in the blood of rabbits.

treated with disulfiram and methanol. No formaldehyde, however, could be demonstrated in these cases although the method of determination is rather sensitive. However, formaldehyde is a very reactive substance and could have been partly bound to the proteins or other substances or metabolized via other routes not sensitive to disulfiram. The marked increase in the toxicity of formaldehyde after disulfiram observed by Owe-Larsson and Skog (15) speaks also in favour of the inhibition of formaldehyde metabolism by disulfiram. The abovementioned enzymes which are inhibited by disulfiram can also oxidize formaldehyde, although rather slowly. Strittmatter and Ball (17) have recently demonstrated in beef and chicken liver preparations a specific formaldehyde dehydrogenase, which is dependent on diphosphopyridine nucleotide and glutathione. The effect of disulfiram on this enzyme and also on the metabolism of methanol in vitro is under study and will be reported upon later.

#### SUMMARY

The effect of disulfiram (tetraethylthiuram disulphide, antabuse) on the elimination rate of methanol from blood was studied in rabbits.

The elimination rate of methanol at a blood methanol concentration of more than about 1000  $\mu\text{g}$  per ml was about 0.0010 mg per ml per minute, and at lower concentrations about 0.00040 mg per ml per minute.

The elimination of methanol from the blood in rabbits previously given disulfiram was considerably retarded.

No formaldehyde was detected in any blood samples of the rabbits given disulfiram, methanol, or disulfiram and methanol.

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STUDIES IN THE EPIDEMIOLOGY OF POLIOMYELITIS  
IN AN ISLAND COMMUNITY<sup>1</sup>

I

PATTERN OF THE POLIOMYELITIS EPIDEMIC ON THE AALAND ISLANDS  
IN 1953

by

N. OKER-BLOM

(Received for publication March 6, 1956)

The present concepts of the epidemiology of poliomyelitis have recently been reviewed by Paul (8). Among other things he calls attention to some epidemics on relatively isolated islands in French Oceania (10). Island epidemics seem to have quite a special pattern, and Gear (2) has lately summarized some of the features of these epidemics.

When in the late spring and summer of 1953 a poliomyelitis epidemic occurred on the Aaland islands, half-way between Finland and Sweden, an opportunity to study an island epidemic in the temperate zone more closely was afforded. It was hoped that virological and immunological studies in this well-defined area would add to the knowledge regarding some intricate and much discussed problems concerned with the epidemiology of the disease, such as the distribution of virus in a community during an epidemic and especially in interepidemic periods, as well as the

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<sup>1</sup> Preliminary reports on parts of this investigation have been given on the XI Scandinavian Congress for Pathologists in Aarhus, Danmark, June 19—23, 1955 (4) and on a meeting of Finska Läkaresällskapet, Helsingfors, Sept. 29th, 1955.



development of antibodies in the population, and consequently the question of the inapparent infection rate during a poliomyelitis epidemic.

The investigation was favoured by the fact that the development of the tissue culture method for the study of the poliomyelitis virus by Robbins, Weller and Enders (9) in 1952 made it possible to carry out studies in this field without using monkeys, which are difficult to obtain in this country.

Altogether 570 stool specimens and 772 sera were collected in this region during the summer 1953, the late spring and summer 1954 and the spring 1955. For the period 1953 to 1954 altogether 144 matched sera were obtained. For practical reasons, however, the collection of specimens could not be started until 10 out of the 59 reported cases of poliomyelitis had already occurred, and for the same reasons the method of random sampling could not be used. Some technical difficulties have delayed the investigation of the specimens which may also have a bearing on the results.

The investigation does not therefore give as good a picture of the state of affairs as was desired, but because some information concerning the spread of the epidemic on the islands and the ratio of apparent to inapparent infections was obtained, the main results of the investigation will be presented in this and two subsequent papers (5, 6), this paper dealing with the main features of the epidemic.

#### THE EPIDEMIC

The Aaland Islands are situated in the Baltic half-way between Sweden and Finland. The archipelago which includes about 6,000 bigger and smaller islands has a population of 22,185 persons. The capital, and the only city of the islands, Mariehamn, is situated on the main island and has 3,590 inhabitants.

Cases of poliomyelitis have occurred on the islands sporadically since the end of the 19th century (3) but there have been no epidemics until in 1953. The number of cases of poliomyelitis on the islands for the years 1915 to 1955 compared to the number of cases in the whole country from 1937 are given in Figure 1.

During the epidemic in 1953 altogether 59 cases diagnosed as poliomyelitis were recorded. Of the cases 23 were paralytic and 39

non-paralytic. From 8 out of 17 paralytic and from 8 out of 26 non-paralytic cases poliomyelitis virus of type 1 was isolated (5).

The distribution of cases in different regions and in different age classes are recorded in Table 1 and Figure 2. In regard to the different environmental living conditions the population has been divided into three main groups, one consisting of persons living in the city, a second consisting of persons living in the rural areas on

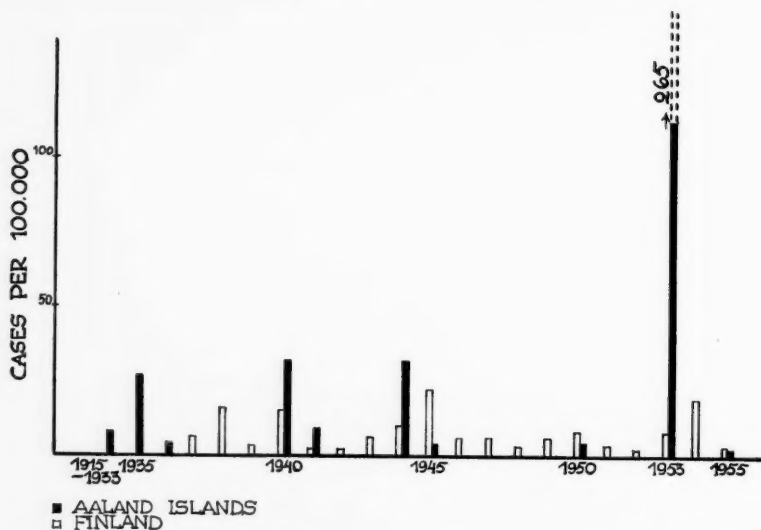


Fig. 1. — Annual number of poliomyelitis cases per 100,000 population in the whole country from 1937 and in the Aaland islands, 1915—1955.

the main island and finally a third consisting of persons living on the smaller surrounding islands.

No paralytic cases occurred in the city. The number of cases in the rural areas on the main island compared to the number of cases on the surrounding islands was 3: 2. The cases were fairly evenly distributed in all the age groups up to 40 years of age, and nearly twice as many paralytic cases occurred in the age groups over 15 years of age. If counted per 1,000 inhabitants the highest incidence of paralytic cases seems to have been in the age group 15 to 20 years. The mortality was 11 per cent.

Two cases occurred in January. Both cases were clinically diagnosed as poliomyelitis and one of them ended fatally. The

TABLE 1

DISTRIBUTION OF PARALYTIC AND NON-PARALYTIC POLIOMYELITIS CASES IN DIFFERENT AREAS AND AGE GROUPS

	Population	Cases of Poliomyelitis			Age Distribution				
		Paralytic	Non-Paralytic	Paralytic per 1000	Paralytic / Non-Paralytic				
					1—9	10—14	15—20	21—40	41—
Main island									
Urban	3,590	0	7	0	0/2	0/3	0/0	0/0	0/2
Rural	14,138	19	24	1.4	5/8	3/4	3/3	6/6	2/3
Surrounding islands	4,457	4	5	0.9	0/2	0/1	1/1	3/1	0/0
Total	22,185	23	36	1.1	5/12	3/8	4/4	9/7	2/5

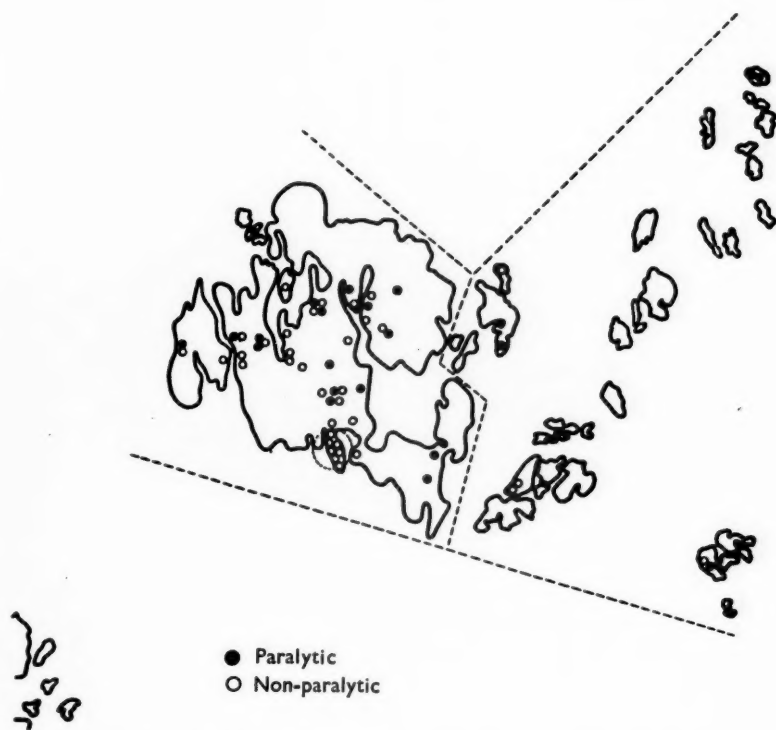


Figure 2. — Distribution of paralytic and non-paralytic poliomyelitis cases in the Aaland islands during the poliomyelitis epidemic in 1953.

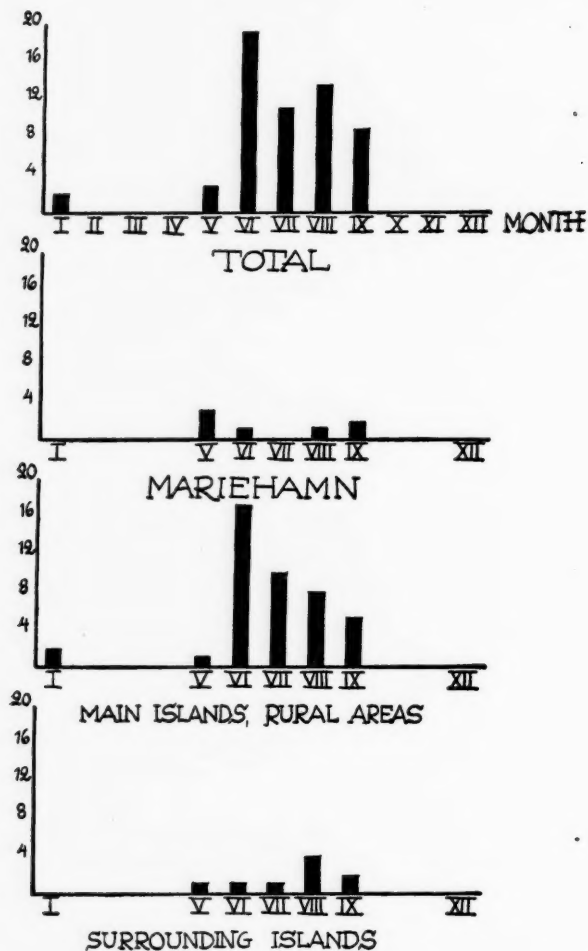


Fig. 3. — Distribution of paralytic and non-paralytic poliomyelitis cases in different regions in the Aaland islands during the poliomyelitis epidemic in 1953.

diagnosis was not verified virologically. The real epidemic started, however, at the beginning of May, culminated at the beginning of July and ended in September (Figure 3). Most of the cases on the surrounding islands occurred in August and September.

The cases were scattered all over the main island but with a concentration to the more populated parts (Figure 2). On some of the bigger surrounding islands no cases whatsoever were recorded.

The two cases occurring on one of the smaller islands in the north-east deserve especial attention in so far that one of the patients had, two weeks before he felt ill, returned from a trip to Sweden, which at that time was swept by an epidemic of poliomyelitis. On his way home he also passed Mariehamn. His nearest neighbour fell ill three days later.

It is difficult to follow the spread of the epidemic, but there seems to have been a spread from the city to the rural areas on the main island and from the main island to the surrounding islands.

The epidemic may, of course, have been caused by an endemic strain, but considering the fact that Sweden was swept by a severe epidemic of poliomyelitis in 1953 (1, 11) and that very few cases of poliomyelitis occurred in other parts of Finland in 1953, it seems reasonable to assume that virus was brought to the islands from Sweden.

In the late summer and fall of 1954 there appeared several cases of meningoencephalitis without paralyses, but none of them could neither clinically nor virologically be diagnosed as poliomyelitis. These cases probably represent cases of the so called »Kumlunge disease», which has occurred endemically on some of the islands in this region, and which apparently is caused by a virus belonging to the RSSE — Louping Ill group (7).

In 1955 one case of paralytic poliomyelitis was recorded in the northern part of the main island.

#### SUMMARY

In the late spring and summer of 1953 the Aaland islands were swept by a type 1 poliomyelitis epidemic. The paralytic attack rate was 105 per 100,000 and the total attack rate 265 per 100,000. Mortality was 11 per cent.

The cases were fairly evenly distributed among persons in the different age classes up to 40 years of age.

The epidemic seems to have spread from the city and the main island to the surrounding islands in the east which were reached by the epidemic about two months later.

It is assumed that the virus was brought to the islands from Sweden, which in the same year was swept by a severe poliomyelitis epidemic of type 1.

A further discussion of some features of the epidemic will be presented in the two subsequent papers on this series (5, 6).

Thanks are due to Dr Kasper Sjöblom and Dr Hannes Koch, as well as all the other colleagues on the island who have kindly made the material available for this study.

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## STUDIES IN THE EPIDEMIOLOGY OF POLIOMYELITIS IN AN ISLAND COMMUNITY<sup>1 2</sup>

### II

#### DISTRIBUTION OF VIRUS IN A COMMUNITY DURING AND AFTER AN EPIDEMIC

by

N. OKER-BLOM and HELENA STRANDSTRÖM<sup>3</sup>

(Received for publication March 6, 1956)

The distribution of poliomyelitis virus in a community both during epidemics and in the interepidemic period has been studied by several authors partly by means of virus isolations from healthy persons (1, 3, 5) and partly by means of isolation of the virus from sewage (9, 10). In a review on the subject Francis concludes that the distribution of the virus tends to be quite focal in character, with epicenters about the households of cases (3).

In the foregoing paper the main features of the poliomyelitis epidemic on the Aaland islands in 1953 were summarized (12). The epidemic apparently spread from the city to the rural areas on the main island and later to the surrounding islands, and therefore an opportunity for a study of the distribution of virus both

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<sup>1</sup> A preliminary report on the investigation presented in this paper was given at a meeting of Finska Läkaresällskapet sept. 29th, 1955.

<sup>2</sup> This investigation was aided by grants from the Following Foundations: «Sigrid Juselius Stiftelse», «Finska Läkaresällskapet» and «Samfundet Folkhälsan i Svenska Finland».

<sup>3</sup> With the technical assistance of Mrs. Vivi Perttunen to whom the authors wish to express their sincere thanks.



in already affected areas as well as in areas so far not affected by poliomyelitis was offered. Attempts were also made to investigate the probable presence of virus during the non-epidemic period in the following years.

As mentioned in connection with the description of this epidemic two cases occurred in January, and the epidemic started at the beginning of May. The collection of specimens did not, however, start until the middle of June. At that time 10 cases had already occurred. All the stool specimens were collected within about two weeks and thus the last specimens were obtained at the time the epidemic had reached its peak.

Stool specimens were collected both from patients and from members of the patients households as well as from healthy persons from regions where at the time of the sampling so far no cases of poliomyelitis had been recorded. This was especially the case in the more isolated islands in the archipelago. In 1953 stool specimens were collected from altogether 403 persons representing about 5 per cent of the population. In the late spring and summer of 1954 specimens were again collected from 167 persons, especially on the surrounding islands, to find out if the virus was still present in the community, and in 1955 finally stools were collected from privies on several places on the islands. Because of technical difficulties most of the specimens were not tested until during 1955.

#### METHODS

*Collection of Specimens.* — Depending on transportation difficulties some of the specimens were stored for a few days at room temperature before they reached the laboratory. In the laboratory the specimens were stored in an electrical deep-freeze at  $-10^{\circ}\text{C}$  to  $-18^{\circ}\text{C}$  until investigated.

*Virus Isolations.* — Roller tube cultures of human embryonic fibroblasts were used for the isolation of virus in the main according to the method described by Robbins, Weller and Enders (16). The typing of isolated cytopathogenic agents was usually performed in cultures of strain HeLa cells *ad modum* Syverton and Scherer (19). Such a method for isolation and typing has recently been described by Svedmyr and coworkers (18).

*Human Fibroblast Cultures.* — Lung tissue from human embryos 3 to 5 months of age were usually employed. If the embryos were even smaller, both lung, skin and muscle tissue of the embryo were used. The fibroblast cultures were set up according to a technic similar to that described by Robbins and coworkers (16) and later by Wesslen (20). The method has been described in detail by Lapinleimu (8) in this laboratory. The nutrient fluid employed was beef amniotic fluid with the addition of 0.05 mg crystalline soybean trypsin inhibitor, 50 I.U. of penicillin and 50  $\mu$ g of streptomycin per ml. The cultures were incubated in a roller drum at 36° C. The medium was changed after 2 to 4 days or immediately before inoculation and thereafter every 4th day.

*HeLa Cell Cultures.* — The cells were maintained in 1 liter Roux flasks by a technic described by Scherer, Syverton and Gey (17) and Syverton and Scherer (19). For the preliminary outgrowth of cells a medium consisting of 50 per cent human serum, 2 per cent chicken embryo extract and 48 per cent Hanks balanced salt solution with the addition of 50 UI of penicillin, 50  $\mu$ g of streptomycin and 20 units of Mycostatin<sup>1</sup> per ml medium was used. Each Roux flask was inoculated with 70 ml of a suspension of 80,000 to 100,000 cells per ml of the above medium. The flasks were kept at 36°C for 7 to 8 days. At this time usually good outgrowth of cells had occurred. The nutrient fluid was removed and replaced by Hanks balanced salt solution containing 0.5 per cent of trypsin (Difco). The bottles were tilted back and forth and as soon as the cells were liberated from the bottom of the flask the suspension was transferred to centrifuge tubes, the cells were thoroughly mixed with a serological pipette and centrifuged for 15 minutes at 1,000 r.p.m. The cells were washed twice with Parker 199<sup>2</sup> and then resuspended in the nutrient fluid to a concentration of 80,000 to 100,000 cells per ml. The counting of cells was performed in a Türk haemocytometer.

The obtained cell suspension was either inoculated into new Roux flasks as above or into tubes in amounts of 0.4 ml. The tubes

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<sup>1</sup> In the beginning of the work some contamination with fungi occurred. Through the courtesy of Dr H. Blank Squibb Institute for Medical Research, E.R. Squibb & Sons, N.J. U.S.A. the antifungal preparation Mycostatin was obtained and since then no contamination with fungi has occurred.

<sup>2</sup> Manufactured by the Pharmaceutical House «Orion», Helsinki.

were pyrex tubes  $130 \times 10$  mm. Tubes were incubated at  $36^{\circ}\text{C}$  in a stationary position. After 3 to 4 days of incubation the tubes were washed twice with 3 ml of Hanks balanced salt solution and refilled with 1 ml of Parker 199 containing 5 per cent inactivated horse serum and the above mentioned antibiotics. These tubes were used for the neutralisation tests.

*Technic for Virus Isolation.* -- A 10 per cent stool suspension was prepared in beef amniotic fluid containing 500 IU of penicillin, 500  $\mu\text{g}$  of streptomycin and 100 U of mycostatin per ml. The stool suspension was shaken in a centrifuge tube containing glass beads and was then centrifuged in a PR1 angle centrifuge at 2,500 r.p.m. at  $5^{\circ}\text{C}$  for 20 minutes. The supernatant was centrifuged once more in the same centrifuge at 15,000 r.p.m. for 15 minutes. The second supernatant was used for inoculation of three fibroblast culture tubes, each tube receiving 0.1 ml. In a few instances only greater inocula according to the method described by Enders and by Kibrick, Enders and Robbins (2, 5) were used.

The tubes were inspected microscopically every forth day for cytopathic changes. As soon as such changes occurred a second passage in fibroblast cultures were made, and if still positive the culture fluid in amounts of 0.1 ml was transferred to three HeLa cell culture tubes. The agents which were positive in the HeLa cell cultures were typed with specific poliomyelitis immune sera. A pool of the three type sera was included to each test. The sera used were mostly monkey sera<sup>1</sup>, but for some typings hyperimmune guinea pig sera prepared in this laboratory according to the method of Gard and his associates (6) were also used. The monkey sera were diluted 1: 50 and in the pool 1: 60. The guinea pig sera which had a titer of 1: 100 to 1: 400 were diluted 1: 5 to 1: 10. The virus serum mixture was kept at room temperature for 1 hour before being inoculated in amounts of 0.1 ml into two HeLa cell tubes. A virus control without serum was included to each test. The tests were read after 2 to 5 days of incubation. The results were usually clear cut and already after 2 to 3 days of incubation, but if not, the virus suspension was titrated and 100 CPD<sub>50</sub> of virus used in neutralisation tests with the immune sera.

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<sup>1</sup> Samples of monkey sera have been kindly supplied by Dr. J. Melnick, Yale University, School of Medicine, New Haven, Conn., U.S.A. Standard reference sera obtained from NFIP have occasionally been used.

The cytopathogenic agents which did not after two to several passages in HeLa cells induce changes were regarded as belonging to the «orphan» group of viruses.

## RESULTS

Among the stool specimens investigated for the presence of the virus altogether 46 cytopathogenic agents were isolated. Of these 35 were diagnosed as poliomyelitis viruses belonging to type 1. The 11 remaining cytopathogenic agents isolated have not been further investigated, but according to their behaviour in fibroblast cultures and in HeLa cells the greatest part of them apparently belong to the group of «orphan» viruses.

In this laboratory several strains of such agents have during 1954 and 1955 been isolated. Some of them have been passed to suckling mice, but with negative results, and they do not behave as viruses belonging to the APC group of which also several strains have been isolated in 1955. In this connection it may be mentioned that 50 of the stool specimens collected in 1953 were investigated for the presence of Coxsackie virus by Pohjanpelto (15), but no virus could be isolated.

The distribution of the isolated poliomyelitis viruses is shown in table 1.

TABLE 1

DISTRIBUTION OF POLIOMYELITIS VIRUS (TYPE 1) AMONG PATIENTS AND HEALTHY PERSONS DURING THE POLIOMYELITIS EPIDEMIC ON THE AALAND ISLANDS IN 1953

	Number tested				Number positive				Per cent positive			
	-10	11-20	21-	Tot.	-10	11-20	21-	Tot.	-10	11-20	21-	Tot.
Patients												
paralytic	5	5	5	17	3	3	2	8	60	50	33	47
non paralytic	10	8	8	26	4	2	2	8	40	25	25	31
Healthy persons												
Main island												
urban	8	17	58	85	1	0	2	3	13	0	3	3.5
rural	57	36	120	213	8	1	4	13	14	3	3	6.0
Surrounding islands	24	13	68	105	0	0	0	0	0	0	0	0.0

It is evident that the percentage of isolations from the patients is low compared to the results obtained elsewhere (2, 7, 11, 18) and also to those obtained in another study in this laboratory (14). In some cases the reason may be that the specimens were obtained too late during the course of the disease (2, 18). Such cases are, however, very few in this material.

Another probable reason may be that the specimens were stored too long and partly inadequately before testing thus resulting in inactivation of virus (4, 14).

Among healthy persons the virus was isolated in 16 cases only (table 1). Of these persons 10 were children below ten years of age, and 10 out of the 16 positives were obtained among family contacts.

The distribution of virus thus shows very much the same pattern as that described by Francis (3) and later by Brown and his associates (1).

The greatest number of positives were obtained in the rural areas close to the city, and no virus could be isolated from persons on the surrounding islands although the percentage of persons tested were about the same in all the three regions. This finding supports the view that at this time the epidemic had not reached the islands in the east.

In 1954 three strains of poliomyelitis virus belonging to type 1 were isolated, one of them from a person on the main island and two from persons on the surrounding islands. The single person on the main island who harboured virus was so far interesting that she belonged to the patients who had been diagnosed as non-paralytic poliomyelitis in 1953, and from whose stools no virus could be isolated at that time. Neither could an increase in antibodies between 1953 and 1954 be shown. It is therefore discussable if she really had a poliomyelitis in 1953. In 1954 she nevertheless had a subclinical infection.

Of the remaining two one was from one of the surrounding islands where one paralytic and one non-paralytic case of poliomyelitis was recorded in 1953, and the other from one of the bigger surrounding islands where no cases of poliomyelitis were recorded in 1953.

In the next year, or in 1955, only several privies on the surrounding islands were investigated for the presence of virus, but no virus could be traced.

Cytopathogenic agents other than poliomyelitis were isolated from the patients in 1953 in one case only. From stools obtained from healthy persons in the city and in the rural areas on the main islands, however, three such agents were isolated in 1953. In 1954 again seven such agents were isolated from stool specimens collected on the surrounding islands but not on the main island. No attempts to connect this agents to any possible clinical entirety have so far been made.

#### SUMMARY

During an epidemic of poliomyelitis on the Aaland Islands in 1953, with a paralytic attack rate of 105 per 100,000, attempts were done to investigate the distribution of virus in the community.

The poliomyelitis virus of type 1 was isolated from only 47 per cent of the paralytic cases and from 36 per cent of the non-paralytic cases. The low percentage of positive isolations has been discussed and it has been suggested that, apart from the too small inocula employed one of the reasons may be the long and partly inadequate storage of the specimens. Considering this fact, the number of isolations from healthy persons shows that the virus was widely disseminated in the population. From 401 stool specimens collected just before or about the time the epidemic reached its peak 16 strains of poliomyelitis virus of type 1 were isolated. Ten out of the 16 strains were isolated from children below 10 years of age and 10 of the strains from familial contacts.

In the interepidemic period in 1954 poliomyelitis virus of type 1 was isolated from 3 out of 167 stool specimens, showing that, although no cases of poliomyelitis occurred in this year, virus was still present in the community. In 1955 several privies in the region were tested for the presence of the virus, but no virus could be traced.

In the previous paper it was assumed that there was a spread of the epidemic from the city to the rural areas on the main island and from the main island to the surrounding islands in the east. This assumption is confirmed by the study of the distribution of virus in the community because when the epidemic had reached its peak and the virus was isolated from several healthy persons on the main island, no virus could be found in the specimens collected from altogether 105 persons on the surrounding islands.



Apart from the poliomyelitis virus, 11 cytopathic agents, apparently belonging to the group of the so-called »orphan» viruses, were isolated during 1953 and 1954.

We wish to thank Dr. J. T. Syverton and Dr. W. F. Scherer who have kindly supplied the strain HeLa cells, Dr. J. L. Melnick who has provided the type sera used and Dr. H. Blank and the Squibb Co. for the readiness to supply the Mycostatin. Finally we wish to express our thanks to Miss M. Blom and Miss A. Holm who have carried out most of the collection of specimens.

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## STUDIES IN THE EPIDEMIOLOGY OF POLIOMYELITIS IN AN ISLAND COMMUNITY

### III

#### DEVELOPMENT OF NEUTRALISING ANTIBODIES AGAINST THE THREE TYPES OF POLIOMYELITIS VIRUS DURING AN EPIDEMIC<sup>1, 2</sup>

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(Received for publication March 6, 1956)

Studies of the distribution of antibodies against poliomyelitis in different population groups have attracted much attention especially since the introduction of the tissue culture method.

By means of these studies valuable informations of the immunity status in different parts of the world have already been and will be obtained (16). Studies of this kind are also likely to elucidate the question of the inapparent infection rate during epidemics and in the interepidemic period. A direct study on the development of antibodies against the three types of poliomyelitis during an epidemic period was performed by Melnick and Ledinko in 1953 (11) by testing matched sera obtained before and after the epidemic.

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<sup>1</sup> A preliminary report on the results presented in this paper was given at the XI Congress for Pathologists in Aarhus, June 19–22 1955.

<sup>2</sup> This investigation was aided by grants from the following Foundations »Sigrid Juselius Stiftelse», »Presidentti J. K. Paasikiven ja Tohtori Wenner-Grenin Lapsihalvaustutkimus Rahasto» »Finska Läkare sällskapet» and »Samfundet Folkhälsan i Svenska Finland».

<sup>3</sup> With the technical assistance of Miss Karin Sandelin to whom the authors wish to express their sincere thanks.

The epidemic of poliomyelitis in the Aaland islands described in the two foregoing papers (12, 14) offered an opportunity for a similar study. This paper deals with the immunity status of the population on the Aaland islands during and after the epidemic.

The serum specimens were collected at the same time as the stool specimens (14), and thus not before, but during the epidemic. In a few instances only specimens were obtained from areas where so far no cases of poliomyelitis had been reported. This was especially the case on the surrounding islands.

As mentioned in the first paper of this series, there was no time to perform the sampling by using a random selection. Attempts were made, nevertheless, to make the specimens as representative as possible for the community. At this time of the year the schools were closed and the children were difficult to reach. The collection of specimens was done by ambulatory nurses and thus difficulties were met with also in obtaining blood specimens from very small children. The fact that the population consists mostly of farmers and fishermen made it difficult to reach whole families and especially to reach the same persons a following year.

Thus, in spite of the efforts, the specimens obtained were not as representative as desired. Nevertheless 342 specimens were collected in 1953. In 1954 the number was 244 and in 1955 again 184 or 772 in all. For the period 1953 to 1954 matched sera were obtained from altogether 144 persons.

Because of the difficulty to obtain specimens from children the persons were divided into three age groups only, viz. those of < 1—20 years, 21—40 years and < 40 years.

#### METHODS

*Serum Specimens.* — The blood specimens were kept for one day at 4°C; the serum was separated by centrifugation, inactivated at 56°C for half an hour and stored in an electrical deep-freeze at —10°C to —18°C. For lack of space in the deep-freeze the sera were later stored at 4°C.

*Neutralization Tests.* — All the neutralization tests were performed in HeLa cell cultures which were prepared according to the methods given in the section for virus isolations (14).

TABLE 1

INCIDENCE OF SERA SHOWING TYPE 1 ANTIBODIES IN THE DILUTION 1:4 OR HIGHER IN THE DIFFERENT AGE GROUPS IN DIFFERENT AREAS IN THE YEARS 1953, 1954 AND 1955.

	Age Groups	1953			1954			1955		
		Number Tested	Number Positive	Per Cent Positive	Number Tested	Number Positive	Per Cent Positive	Number Tested	Number Positive	Per Cent Positive
Mariehamn	1—20	11	4	36±15	4	1	25±22	57	36	63±6
	21—40	69	41	59±6	19	14	74±10	40	19	48±8
	41—	44	26	59±6	11	7	64±15	23	16	70±10
Main island rural areas	1—20	45	23	51±7	22	15	68±10	40	31	78±7
	21—40	42	25	60±8	23	19	83±8	6	6	100
	41—	42	37	88±5	21	20	95±5	3	2	67±27
Surrounding islands	1—20	19	3	16±8	23	5	22±9	15	8	53±13
	21—40	34	21	62±8	43	24	56±8			
	41—	36	30	83±6	78	65	83±4			
		342			244			184		

The virus strains used for the neutralization tests were, for type 1 a Brunhilde strain, for type 2 the MEF 1 strain and for type 3 the Saukett strain. The virus strains were kindly supplied by dr. T. Wesslen at the State bacteriological laboratory Stockholm, Sweden.

The CPD<sub>50</sub> of the virus used for the tests was determined by titrations using 0.5 log dilutions for the virus in 8 HeLa tubes for each dilution.

100 CPD<sub>50</sub> of virus per 0.1 ml was mixed with serum diluted 1:4, both in amounts of 0.2 ml. The virus-serum mixtures were kept at room temperature for 1 hour and inoculated into two HeLa cell tubes, each receiving 0.1 ml. The tests were microscopically read after 5 days of incubation at 36°C. Antibody titrations were performed by using fourfold dilutions of serum and otherwise as stated above.

Each serum was tested for cytopathic effect in the same dilution without virus and each day a virus control consisting of 50 CPD<sub>50</sub> of virus in two HeLa cell tubes were included.

## RESULTS

Because the epidemic was of type 1, all the sera were tested for antibodies against this type (table 1) and the matched sera only tested for antibodies against all the three types of virus (table 2). When divided into groups according to age and dwelling place several of the groups turned out to be very small. Thus it is difficult to draw any conclusions from the differences between some of the groups. Nevertheless some information can be obtained.

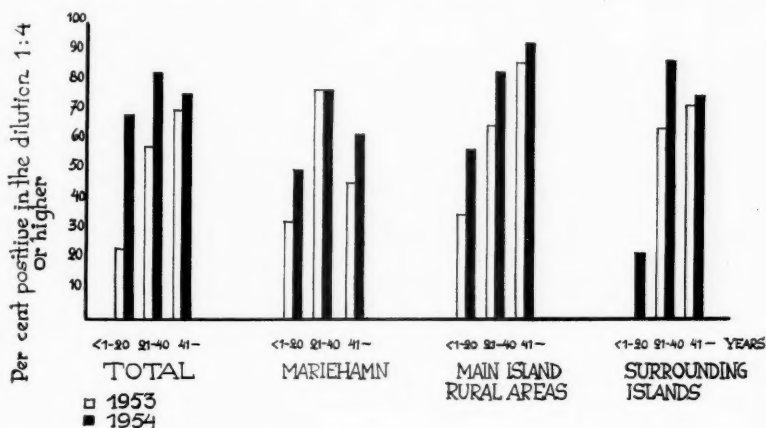


Fig. 1. — Serological response for Type 1 poliomyelitis virus in different age groups and areas.

If all the sera tested for type 1 antibodies only are dealt with, there is a tendency towards a higher incidence of antibodies in the higher age groups, as expected. Especially as far as the persons living on the surrounding islands are concerned this difference is typical and statistically significant. The incidence of persons having type 1 antibodies in the age group of <1—20 years on the surrounding islands is very low compared to the incidence in the same age group in the city and in the rural areas on the main island. Between the two latter groups the difference is highly significant.

An increase in the incidence of positives from 1953 to 1954 was observed in the age groups of <1—20 years and 21—40 years in the rural areas on the main island and in the age group of <1—20 years on the surrounding islands, but the difference is statistically significant only in the age group of 21—40 years in the rural area

TABLE 2

INCIDENCE OF NEUTRALIZING ANTIBODIES IN THE SERUM DILUTION 1:4 AGAINST THE THREE TYPES OF POLIOMYELITIS VIRUS IN DIFFERENT AREAS IN 1953 AND 1954

Area	Age Groups	Number Tested	1953			1954		
			Per Cent Positive			Per Cent Positive		
			Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
Mariehamn	<1—20	6	33			50		
	21—40	17 <sup>1</sup>	77	69	54	77	69	54
	41—	13	46	69	54	62	69	54
Main island rural areas	<1—20	14	35	35	35	57	43	43
	21—40	17	65	59	41	83	59	41
	41—	14	86	79	86	93	79	86
Surrounding islands	<1—20	9	0	67	44	22	67	44
	21—40	22	64	82	46	86	82	46
	41—	32	72	84	81	75	84	81
Total	<1—20	29	24	53	35	69	58	39
	21—40	56	68	71	46	82	71	46
	41—	59	70	80	78	76	80	78

<sup>1</sup> In this group only 13 sera were tested for type 2 and type 3 antibodies.

on the main island. The figures for 1955 are unfortunately too small for any conclusions, but there is a tendency towards a further increase in this year in the same groups, especially on the surrounding islands, and probably also in the youngest age group in the city.

If a calculation of the number of infected is performed on the basis of these figures in the rural areas on the main island, there should have been 250 infected per every paralytic case in the age group of 21—40 years considering that after the completion of the sampling there appeared 4 paralytic cases in this group. The corresponding number for the whole rural area on the main island would be 225 inapparent infections per every paralytic case. For the city and for the surrounding islands such a calculation can not be made.

Especially as far as the surrounding islands are concerned this is surprising because, as has been pointed out in the foregoing

TABLE 3

DETERMINATION OF RATIOS OF PARALYTIC CASES TO SUBCLINICAL INFECTIONS FOR DIFFERENT AGE GROUPS AND DIFFERENT AREAS

Area	Age Groups	Population	Paralytic Cases		Case Rate Per 1000		Subclinical Infection Rate		Infections Per Case	
			I Total	II After Samling	I	II	Observed Ratio <sup>1</sup>	Per Cent	I	II
Whole Archipelago	all age groups	22.185	23	13	1.1	0.6	18/58	31	280	<b>520</b>
	<1—20	7.717	12	6	1.5	0.8	6/22	27	180	<b>340</b>
	21—40	5.595	9	6	1.6	1.1	8/18	44	280	<b>400</b>
Surrounding islands	all age groups	4.457	4	3	0.9	0.7	8/26	31	340	<b>440</b>
Main island Rural	all age groups	14.138	19	10	1.4	0.7	7/17	41	300	<b>600</b>

<sup>1</sup> *No. of cases developing antibodies during epidemic*  
*No. of cases without antibodies in pre-epidemic period*

sections of this paper, the epidemic can hardly have reached this region at the time the specimens were collected, and this is therefore one of the few regions which should show an antibody level similar to that before the epidemic. If, as has also been assumed, the epidemic reached these islands later, this should have been reflected in the antibody level of the population. The reason why this is not the case is apparently that the antibody level on different islands varies widely, and, because the specimens collected in 1953 and 1954 are not all collected exactly at the same islands, totally virgin island may have been included in 1954. On the other hand the number of specimens collected, and of persons actually living, on many of these islands are too small to be accounted for separately.

A more reliable picture is therefore obtained from the matched sera several of which were from those very islands. Of the 63 persons on the islands from whom matched sera were obtained 8 showed an increase in antibodies from 1953 to 1954 (table 2 and figure 1). In the rural areas on the main island the corresponding numbers were 7 out of 45 and in the city 3 out of 46. The pro-



TABLE 4  
ANTIBODY TITERS AGAINST TYPE 2 AND TYPE 3 IN SERA SHOWING AN INCREASE  
IN TYPE 1 ANTIBODIES.

Name	Age	Date of Specimen	Antibody Titer		
			Type 1	Type 2	Type 3
M. E.	61	5. 7. 53	<2	<4	
		10. 5. 54	4	4	
E. Ö.	24	4. 7. 53	2	<4	16
		8. 5. 54	64	<4	64
G. G.	18	5. 7. 53	<2	16	<4
		8. 5. 54	64	1.024	<4
B. S.	42	27. 6. 53	4	4	4
		6. 7. 54	64	4	256
L. E.	29	26. 5. 53	<2		<4
		10. 5. 54	4		<4
M. F.	26	25. 6. 53	<2	16	64
		10. 5. 54	64	16	64
A. H.		11. 7. 53	<2	64	64
		12. 5. 54	16	64	64
E. S.		11. 7. 53	<2	<4	<4
		17. 6. 54	256	64	<4
O. C.	70	11. 7. 53	<2		
		12. 5. 54	4		
E. W.	60	27. 6. 53	4		
		11. 5. 54	16		
H. K.	54	11. 9. 53	<2	256	256
		26. 7. 54	8	256	256
S. G.	11	22. 6. 53	2	<4	<4
		27. 7. 54	256	16	16
M. C.	22	25. 6. 53	<2	16	256
		15. 7. 54	2	16	256
E. E.	35	20. 6. 53	<2	4	64
		30. 7. 54	64	4	64
E. H.	49	20. 6. 53	<2	<4	64
		21. 7. 54	256	<4	64
E. J.	40	25. 6. 53	<2	<4	<4
		29. 6. 54	16	<4	<4
C. F.	17	25. 6. 53	<2	<4	<4
		29. 6. 54	256	<4	<4
F. G.	41	29. 6. 53	4	4	64
		15. 7. 54	16	4	64
A. L.	9	27. 6. 53	<2		
		9. 5. 55	256		
B. S.	7	20. 6. 53	<4	<4	<4
		13. 8. 54	16	<4	<4

portion of persons who developed antibodies during the epidemic was 21 per cent in the age group of < 1—20 years, 14 per cent in the age group of 21—40 years and 7 per cent in the age group of over 40 years of age.

These figures are small, but if a calculation of the number of in apparent infections is made according to Melnick and Ledinko (11) and taking into consideration only those paralytic cases which appeared after the sampling, one arrives to the conclusion that the inapparent infection rate for the whole archipelago was 520; in the age group of < 1—20 years it was 340 and in the age group of 21—40 years 400 per every paralytic case (table 3). In the age group of over 40 years only one paralytic case occurred after the sampling, and it may be wise to avoid a calculation. The subclinical infection rate was, when calculated in this manner, higher on the main island than on the surrounding islands being 600 and 440 respectively.

If the calculation is based on the number of persons without antibodies at the time of sampling the figures are 270 in the age group of < 1—20 years and 140 in the age group of 21—40 years, and for the whole archipelago 230 per every paralytic case.

The incidence of antibodies against type 2 were fairly high also in the youngest age group showing an increase with increasing age (table 2). The incidence of antibodies against type 3 was somewhat lower, but significantly higher in the youngest age group, especially on the surrounding islands, than the incidence of antibodies against type 1. There was no significant change in the incidence of type 2 or type 3 antibodies between 1953 and 1954, but a few of the post-epidemic sera showed an increase in the titer for type 2 or type 3 antibodies. For this reason all the sera showing an increase in antibodies against type 1 were also titrated for antibodies against type 2 and type 3 (table 4). In a few sera a marked increase also in these antibodies was observed. Considering the fact that the only type of virus isolated was of type 1, the results speak for a heterotypic antibody response. (16)

#### DISCUSSION

The investigation presented in this and the two foregoing papers was performed with the intention to study the pattern of a poliomyelitis epidemic in an island community. Attempts were

made to follow the distribution of virus during the epidemic, to find out if virus was still present in the community in the following years, and finally to obtain a picture of the inapparent infection rate during such an epidemic.

It was already mentioned in the introduction, and it has in several connections been pointed out that certain technical and practical difficulties have greatly limited the value of both the collected material and the tests themselves.

As far as the material is concerned, a great disadvantage is the shortage of children. A collection of specimens at random would also have given a more reliable picture of the state of affairs. Finally the collection of specimens was started too late. This does not so much affect the question of the distribution of virus because, as Francis (3) has pointed out, virus isolation has the limitation that it in any case gives a view of a given time or for a short period only. In this case it gives a picture of the situation when the epidemic had reached its peak. Far more this fact affects the antibody studies because it is very difficult to know how many had already been infected and had developed antibodies by the time when the sampling started.

The methods for isolations of the virus seem to have given too few positives. As, has recently been pointed out (2, 8, 18), a simple enlargement of the inoculum may have somewhat increased the number of positives, but we feel that the prolonged storage also must have been one of the limiting factors. Finally the possibility cannot be totally excluded that some of the cases were not poliomyelitis. The so called »Kumlinge disease» which has been shown to be a tick-borne meningoencephalitis belonging to the RSSE-Louping Ill group (13) and which some times has a clinical picture fairly similar to non-paralytic poliomyelitis has occurred endemically in this areas. It is quite probable that at least some of the non-paralytic cases, as for instance the one having virus in her stools in 1954, was of this type.

Considering these facts conclusions must be drawn with care. Nevertheless there are some results which, with a certain degree of probability, can be pointed out.

All the results are in agreement as far as the spread of the epidemic is concerned. Thus the whole archipelago, with the exception of few isolated islands, was swept by a type 1 epidemic,

which started in the city, or in the rural area on the main island close to the city, from which it spread to the outskirts of the main island and finally to some of the surrounding islands in the east.

When the epidemic had reached its peak on the main island and virus was isolated from several healthy persons in this area no virus could be traced on the surrounding islands. The antibody level on the surrounding islands was at that moment far below that on the main island. It would be tempting to assume that the higher incidence of type 1 antibodies on the main island depends on the fact that a considerable number of people had already been infected and had developed antibodies and that the real immunity status of the whole archipelago before the epidemic was somewhat similar to that on the surrounding islands. This assumption may be supported by the fact that the immunity status for type 3 was nearly exactly the same in all the regions and that the incidence of type 2 antibodies was somewhat higher on the surrounding islands, showing that the exposure to these types had been about the same and probably somewhat higher on the surrounding islands.

Nevertheless the incidence of antibodies against type 1 in the age group of < 1—20 years is very low on the surrounding islands. It may be mentioned in this connection that the corresponding figures for Helsinki and for some other parts of the country (9) as well as for some parts of Sweden (15) are about three times higher.

The distribution of the virus in the community is very much the same as that described by Francis (3) from several epidemics. Thus there was an accumulation of virus to those households where cases of poliomyelitis had occurred, and, at the whole, an accumulation of virus to family contacts, but still virus was found also from healthy persons who had had no known contact with the patients. The results also show that virus carriers were found especially among children, and therefore it would have been very desirable if more children could have been included in the material. Nevertheless these results support the view held by Francis that «the distribution of poliomyelitis virus tends to be focal in character with epicenters around the households of cases».

The attempts to isolate virus during the epidemic-free periods in 1954 and 1955 show that although no cases whatsoever occurred, the virus was still in the community. It is difficult to say if this

virus was imported from other parts of the country, because an epidemic of type 1 swept the southern part of the country in 1954. However, the fact remains that compared to 16 out of 401 persons in 1953, 3 out of 167 persons harboured virus in 1954 in spite of the fact that no cases of poliomyelitis occurred in 1954. Two of the three persons were from the surrounding islands and it is noticeable that there was a tendency towards an increase in type 1 antibodies both on the main island, but especially on the surrounding islands also for the period 1954 to 1955.

Attempts to calculate the inapparent infection rate have been made partly on the basis of the increase in the incidence of type 1 antibodies as determined for all the sera tested in 1953 and 1954 and partly on the basis of the results obtained with the matched sera.

The number of inapparent infections is usually given as something between 100 and 350 for every paralytic case (1, 4, 7, 10, 16).

The figures obtained here are higher than those obtained by Melnick and Ledinko (9). When the number of persons developing antibodies during the epidemic are computed against the total number of paralytic cases the figures obtained are, however, very much in accordance with those obtained by Gard (4). It seems logical, however, to calculate the inapparent infection rate on the basis of those paralytic cases which occurred after the sampling was finished, and in such case the figures obtained are still higher than the number of subclinical infections given by Gard (4). It is unfortunate that an exact calculation can not be made for the inapparent infection rate in the lower age groups for instance below ten years of age, but considering the fact that more than 50 per cent of the virus isolations were from children below ten years of age the inapparent infection rate in this group must have been even higher than that given for the whole group of < 1—20 years.

Accordingly the number of inapparent infections for the whole archipelago is somewhere between 230 and 520 per every paralytic case which means that something between 25 and 50 per cent of the population had been exposed to infection at sometime during or after the epidemic. A similar calculation has been made by Gard who studied an epidemic in Sweden (5).

Compared to the epidemics on the mainland the attack rate is very high, but compared to the other island epidemics described (6) the attack rate is low. The archipelago, although it consists of many

very isolated islands, does not represent a virgin population as far as contacts with poliomyelitis virus is concerned, but the distribution of paralytic cases in the different age groups and the antibody studies show that a great part of the population has not for many years been in contact with poliomyelitis virus of type 1.

#### SUMMARY

In the three papers of this series attempts have been made to study the pattern of the poliomyelitis epidemic on the Aaland Islands in 1953.

The whole archipelago, with the exception of some more isolated islands, was swept by a type 1 poliomyelitis epidemic, the paralytic attack-rate being 105 and the total attack-rate 265 per 100,000. The recovery rate of virus from the patients was low being 47 per cent among the paralytic patients and 32 per cent among nonparalytic patients. The distribution of virus in the community at the time the epidemic had reached its peak shows that among healthy persons virus was most frequently isolated from children below 10 years of age and from family contacts. In the year following the epidemic virus of type 1 was again isolated from 3 persons although no verified case of poliomyelitis occurred in this year.

Studies of neutralizing antibodies against the three types of poliomyelitis virus during and after the epidemic showed that the incidence of neutralizing antibodies against type 1 was very low in the younger age groups on the more isolated islands in the epidemic year. When tested after the epidemic an increase in type 1 antibodies in several areas was shown. Calculated on the basis of the immunological data the inapparent infection rate was for the whole archipelago 230 to 520 per every paralytic case, showing that something between 25 and 50 per cent of the population had been exposed to infection at some time during or after the epidemic.

Very small changes was seen in the incidence of type 2 and type 3 antibodies, and those seen were regarded as a heterotypic antibody response.



We wish to express our thanks to Dr. T. Wesslén who has kindly supplied the virus strains used, and to Miss M. Blom and Miss A. Holm who have carried out most of the collection of the specimens.

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## PROPAGATION OF LOUPING-ILL VIRUS IN MALIGNANT HUMAN EPITHELIAL CELLS, STRAIN HELA<sup>1</sup>

by

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A review summarizing the effect of animal viruses on cells cultivated in vitro has been published by Enders in 1954 (5). Of the viruses belonging to the encephalitis group, equine encephalomyelitis viruses have been cultivated in chicken fibroblasts as early as in 1942 (6) and later by several authors (1, 3, 12). Recently Scherer and Syverton (9) have adapted the viruses of Eastern and Western equine encephalomyelitis, west Nile, St. Louis encephalitis and Japanese B encephalitis to malignant human epithelial cells, strain HeLa.

Encephalitides apparently caused by a virus belonging to the Russian Spring Summer Encephalitis (RSSE) — Louping Ill (LI) group have recently been shown to occur in this country (7). Attempts have therefore been made to adapt these viruses also to tissue culture. This paper deals with the propagation of Louping Ill virus in cultures of malignant human epithelial cells strain HeLa.

### MATERIAL AND METHODS

*Virus Strain.* — The strain of Louping Ill virus was kindly supplied by Dr. D. G. ff. Edward, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England. The virus has

<sup>1</sup> Aided by grants from the Sigrid Jusélius Foundation.

<sup>2</sup> The skilful technical assistance of Miss Irja Rouhia is gratefully acknowledged.

since June 1955 been passed intracerebrally in albino mice through altogether 7 passages. When transferred to tissue culture in September 1955 the virus was in its third passage in this laboratory and had a titer of  $10^{-6.7}$ .

*Titration of Virus in Mice.* — A ten per cent brain suspension was prepared by grinding the brain in a mortar with the addition of 50 per cent rabbit serum in phosphate buffer with pH 7.2, containing 50 IU of penicillin and 50  $\mu$ g of streptomycin. The brain suspension was centrifuged at 2,000 r.p.m. for 15 minutes, the supernatant collected and divided into small test tubes which were stored at  $-10^{\circ}\text{C}$  to  $18^{\circ}\text{C}$ . Tenfold dilutions of the virus suspension were prepared in the same phosphate buffer containing 10 per cent rabbit serum and each dilution was injected intracerebrally in amounts of 0.03 ml into 5 mice weighing 9 to 11 grams. The mice were observed for 14 days, and the 50 per cent end point was calculated. — Paralysis and death usually occurred on the 5th to 7th day. — Tissue culture virus was titrated in mice in a similar manner, but instead of the dilution fluid mentioned above, Parker 199<sup>1</sup> with the addition of 10 per cent chicken serum was used.

*Tissue Culture Technic.* — The strain of HeLa cells used was supplied by the courtesy of Dr. Jerome T. Syverton, Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minn. U.S.A. Cultures of HeLa cells were prepared in the main according to the technic of Scherer, Syverton and Gey (10), and has in detail been described in another connection (8). The nutrient fluid consisted of 50 per cent human serum, 48 per cent Hanks balanced salt solution and 2 per cent chicken embryo extract with the addition of 50 IU of penicillin, 50  $\mu$ g of streptomycin and 20 U of Mycostatin.<sup>2</sup> The fluid used for the maintenance of cells was Parker 199 with the addition of in the beginning, 5 to 10 per cent horse serum, and later 10 per cent chicken serum and the above mentioned antibiotics.

Throughout the work flasks of the Carrel type with a diameter of 60 mm and pyrex test tubes  $130 \times 10$  mm were used. The flasks

<sup>1</sup> Manufactured by the Pharmaceutical House «Lääketehtas Orion», Helsinki.

<sup>2</sup> At the beginning of the work some contamination with fungi was seen. Through the courtesy of Dr. H. Blank, Squibb Institute for Medical Research, E. R. Squibb & Sons, New Jersey, U.S.A., the antifungal preparation Mycostatin was obtained. Since then no contamination with fungi has occurred.

received about 300,000—400,000 cells in 4 ml of fluid and the tubes 30—40,000 cells in 0.4 ml of fluid. After the outgrowth of cells the flasks and tubes were washed twice with 10 ml and 3 ml respectively of Hanks balanced salt solution and refilled with 4 ml and 1 ml respectively of the fluid used for the maintenance of cells. Fluid was changed every 5th to 8th day. The cultures were observed microscopically for probable cytopathogenic changes every day.

*Titration of Virus in Tissue Cultures.* — Tenfold dilutions of the tissue culture fluid were made in Parker 199 containing 10 per cent chicken serum and the above mentioned antibiotics. Each dilution was inoculated into two HeLa cell tubes in amounts of 0.2 ml, and the tubes were incubated at 36° C for 6 to 7 days.

*Neutralization Tests in Tissue Culture.* — Specific immune serum was diluted from 1: 100 to 1: 3200 by using twofold dilutions. To 0.35 ml of each serum dilution 0.35 ml of tissue culture virus containing 100 CPD<sub>50</sub> per 0.1 ml was added. The serum-virus mixtures were kept at 36° C for 2 hours and each dilution inoculated into three HeLa cell tubes each tube receiving 0.2 ml.

The dilution fluid used was Parker 199. In the case of virus dilution, we used Parker 199 with the addition of 10 per cent chicken serum.

*Neutralisation Tests in Mice.* — The tests were carried out by intracerebral injections of virus-serum mixtures. Undiluted serum and serial tenfold dilutions of virus were used according to the method given by Smadel (11).

## RESULTS

Even in the first passage some cytopathogenic changes were seen in the HeLa cells. There appeared several groups of shrunken cells, and during incubation the amount of cells clearly diminished compared to the normal controls. The death of cells occurred very slowly, however, and it was not until at the 7th day that a clear difference between cultures inoculated with virus and normal controls was seen. Prolonged incubation up to 15 days resulted in the death of nearly all the cells, but some living cells could still be seen. During passage, however, cytopathogenic changes appeared regularly on the 5th to 6th day, and on the 9th day most of the cells were usually destroyed (Figures 3 to 5).

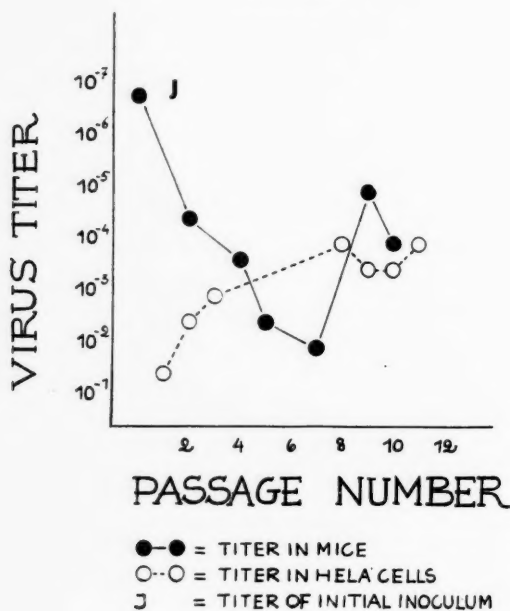
TABLE 1  
GROWTH OF LOUPING III VIRUS IN CULTURES OF STRAIN HELA CELLS

Passage Number	Total Days in Culture	Cumulative Log of Dilution of Initial Virus Inoculum	Infectivity of Cultural Fluid	
			Mice <sup>1</sup>	Hela Cells <sup>2</sup>
Inoculum			6.7	
1	4	2.0		1.5
2	11	4.0	4.5	2.5
3	19	6.0		3.0
4	27	9.0	3.6	
5	33	9.7	2.5	
6	39	10.4		
7	46	11.1	2.0	
8	62	11.8		4.0
9	66	12.5	5.0	3.5
10	77	13.6	4.0	3.5
11	85	15.0		4.0

<sup>1</sup> Negative log of the LD<sub>50</sub>/0.03 ml of diluted cultural fluid.

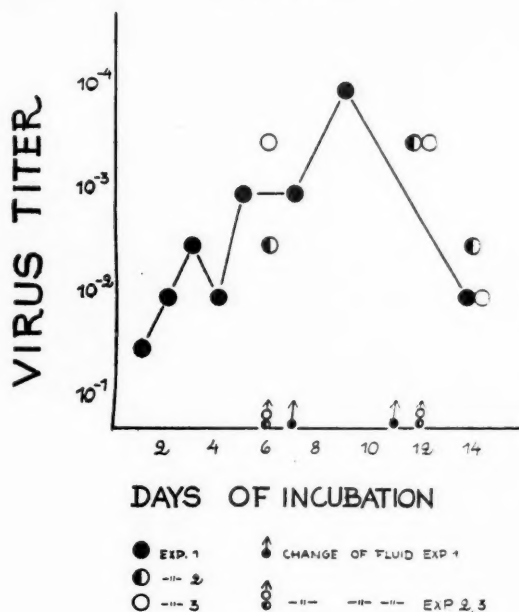
<sup>2</sup> Negative log of the dilution of cultural fluid which produced a specific cytopathogenic effect in Hela cell cultures after 6 to 8 days of incubation at 36°C.

FIGURE 1



To gain information of the multiplication of virus in the HeLa cells, fluid from several passages were titrated in mice and in HeLa cells (Table 1 and Figure 1). Virus infectious for mice and destructive for HeLa cells thus persisted through 12 serial passages over

FIGURE 2



a period of 85 days. The initial virus inoculum with a titer in mouse of  $10^{-6.7}$  was diluted  $10^{15}$  times. Thus the results present evidence that virus has multiplied in the cultures.

This is also shown in an experiment performed with the intention to estimate the growth curve of LI virus in HeLa cells (figure 2). The highest yield of virus was in several experiments obtained at the 7th to 10th day of incubation, apparently at the 8th to 9th day.

It is interesting to note that the titer as estimated in mice during the first passages showed a considerable decrease. In the later passages, however, an increase in the virus titer for mice was again observed.

Neutralization tests both with mouse passage virus and tissue culture virus was performed in tissue culture, as well as in mice,

TABLE 2

COMPARATIVE NEUTRALISATION TESTS IN TISSUE CULTURE AND IN MICE WITH LOUPING III VIRUS OBTAINED FROM MICE AND TISSUE CULTURE

	Virus Dilution						Neutral. Index	Serum Dilution					
	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>		100	200	400	800	1600	3200
Mouse brain susp	5/5 <sup>1</sup>	5/5	5/5	5/5	1/4	0/5	6320						
Mouse brain susp + spec immune ser	1/5	0/5	0/3	0/5	0/5	0/5							
Tissue culture virus	5/5	3/5	0/5	0/5			≥500						
Tissue culture virus + spec immune ser 1:10	1/5	0/5	0/5	0/5									
100 CPD <sub>50</sub> of Tissue culture virus								—	—	—	—	—	+
								—	—	—	—	—	+
								—	—	—	—	+	+

<sup>1</sup> Numerator = number of mice dead  
Denominator = number of mice injected

<sup>2</sup> + = cytopathic changes  
— = no cytopathic changes

by using specific hyper immune sheep serum <sup>1</sup> (Table 2). The neutralization of mouse passage virus, as well as tissue culture virus, by specific immune serum as estimated by neutralization tests in mice and the prevention of cellular destruction by the specific antibody established a causal relationship between LI virus and its cytopathic effect for strain HeLa cells. The experiments also showed that cultures of HeLa cells can be utilized for the demonstration of antibodies against LI virus.

Considering the close antigenic relationship between LI virus and the RSSE virus (2, 4) it has been assumed that the method can be used for the demonstration of antibodies against RSSE virus as well.

Neutralization tests performed on several human sera with the

<sup>1</sup> Lyophilized hyperimmune sheep serum was supplied by Lederle Laboratories Division, Pearl River, N.J., U.S.A. through the courtesy of Dr. H. Cox. and Dr. D. Wolfe, in 1951.

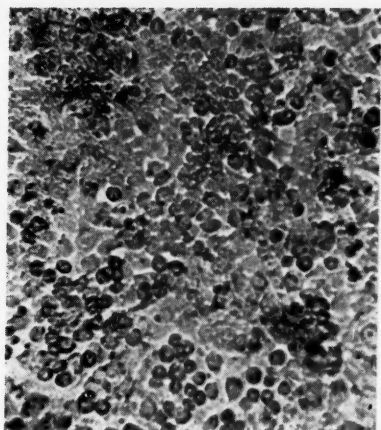


Fig. 3. — Strain HeLa cells kept at 36° C for 8 days in Parker 199 containing 10 per cent chicken serum.

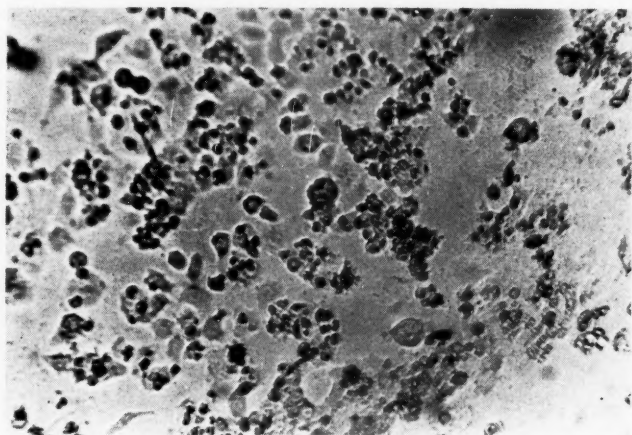


Fig. 4. — Strain HeLa cells photographed 6 days after inoculation of louping ill virus.

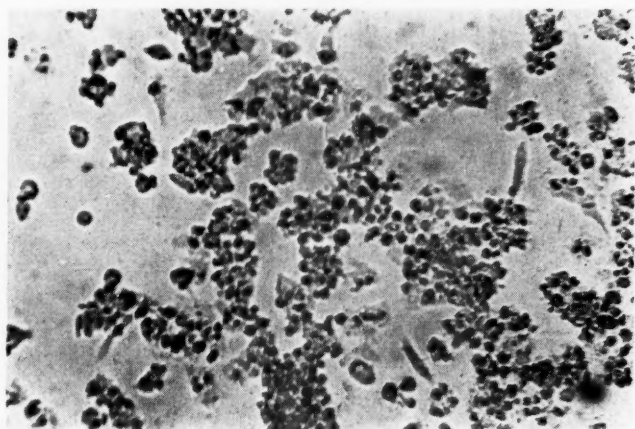


Fig. 5. — Strain HeLa cells photographed 8 days after inoculation of louping ill virus.





intention to investigate the distribution of antibodies against this group of viruses have also shown that a good correlation exists between neutralization tests performed in mice and in tissue culture with the LI virus (7).

#### SUMMARY

Louping Ill virus was successfully propagated in malignant human epithelial cells, strain HeLa. The virus causes clearly demonstrable cytopathogenic changes in the cells after an incubation period of 5 to 6 days.

It was shown that the HeLa cells can be used for the demonstration of specific antibodies against Louping Ill virus, and it has consequently been assumed that the method can be utilized for the demonstration of RSSE antibodies as well.

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## EFFECT OF »TWEEN 80» ON THE NICOTINIC ACID SYNTHESIS OF *ESCHERICHIA COLI*

by

JOUNI JÄNNES

(Received for publication April 3, 1956)

Davis and Dubos (1), Hotchkiss (2) and Valko (3) have described the theoretical basis of the use of detergents in microbiology. It was also noted (3) that some of the detergents may have an inhibitory effect on the enzymatic systems of microbiology. The effect of detergents on the growth of bacteria has been investigated by many authors.

Nevertheless, no investigation concerning the effect of detergents on the vitamin synthesising capacity of bacteria has as yet not been published.

This paper deals with some series of tests made to clarify this problem. Seven series of tests were made, of which three are selected here as the most characteristic. The detergent used in this work was one of the most common »Tween 80» (Polyoxyethylene-sorbitanmonooleate).

The test organism used was a common strain of *Escherichia coli* isolated from human feces.

The medium contained the following substances:

Ammonium tartrate 7 g  
Ammonium sulphate 3 g  
Dibasic potassium phosphate 2 g  
Lactose 5 g  
Water to make 1 litre

Of this medium 50 ml was pipetted into 100 ml Erlenmeyer bottles. One bottle was free from the addition of »Tween 80»; into

the other bottles »Tween 80» was pipetted in different amounts. After sterilisation the bottles were inoculated with a drop of *E. coli* suspension.

After 24 hours' growth at 37° C the samples were centrifugated and the assays of nicotinic acid were carried out from the clear supernatants. Nicotinic acid was estimated microbiologically, using *Lactobacillus arabinosus* 17—5 as test organism. The »basal medium» was that recommended by the American Association of Vitamin Chemists in 1951 (4). To prevent the influence of »Tween 80» on the growth of *Lactobacillus arabinosus* 17—5, »Tween 80» was also added to the »basal medium» in a concentration of 1 ml per litre of medium.

When strain No. 1 was used in the experiments the following results were obtained:

Concentration of »Tween 80» per ml of Medium for <i>E. coli</i>	Turbidity Readings for the Growth of <i>E. coli</i> (Pulfrich Photometer)	Synthesised Nicotinic Acid in $\gamma$ per ml
0.000	0.22	0.04
0.001	0.22	0.03
0.002	0.21	0.02
0.003	0.19	0.02

These experiments also show that the addition of »Tween 80» in a concentration of 0.002—0.003 ml per ml of medium was capable of decreasing the amount of synthesised nicotinic acid by about 50 per cent. It is also obvious from these results that »Tween 80» at first inhibits the ability to synthesise nicotinic acid and that greater amounts of this substance inhibit also the growth of the micro-organism.

Experiments with smaller amounts of »Tween 80» also showed a similar tendency.

The results of these tests were as follows:

»Tween 80» in ml per ml of Medium	Turbidity Readings with Pulfrich Photo- meter	Synthesised Nicotinic acid in $\gamma$ per ml of Medium
0.0000	0.22	0.05
0.0001	0.21	0.04
0.0002	0.22	0.05
0.0003	0.21	0.03

These results were also confirmed by experiments with another strain of *Escherichia coli*.

This strain was also isolated from human feces and its ability to synthesise nicotinic acid was tested before the experiments were made.

The results of these tests were as follows:

»Tween 80» in ml per ml of Medium	Turbidity Readings with Pulfrich Photo- metre	Synthesised Nicotinic Acid in $\mu$ g per ml of Medium
0.000	0.32	0.07
0.001	0.33	0.05
0.003	0.31	0.02
0.005	0.13	none

In this series the author noted that the addition of »Tween 80» in a concentration of 0.005 ml per ml of medium was able to inhibit the growth of *Escherichia coli* very clearly. The results for the nicotinic acid synthesis had the same trend as the earlier results. As postulated by Valko (3), the detergents may in some case have an enzyme-inhibiting effect on micro-organisms. These tests show that »Tween 80» has this effect on the metabolism of *E. coli*. The recommended addition of »Tween 80» to many microbiological media is of a level which may give some importance to the findings described here.

#### SUMMARY

The addition of »Tween 80» in concentration of 0.001—0.002 ml per ml of medium effected a very definite inhibition of nicotinic acid synthesis by normal strains of *Escherichia coli*.

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## A NEW FLOCCULATION TEST FOR PATHOLOGICAL SERA

by

M. TUOMIOJA<sup>1</sup> and P. KAJANNE

(Received for publication April 4, 1956)

In the study on the effect of the addition of various lipids and surface active substances to the VDRL antigen, a series of technical products produced by Atlas Powder Company, Wilmington, Delaware, was tested. With three of these substances there was a flocculation with some pathological sera to be observed if the cardiolipin was replaced by them in the original antigen. These substances were Span 60, Tween 61 and Span 65. These substances are stated to contain a mixture of sorbitan monostearates. The most active of them was Span 60.

A number of various sera are tested according to the VDRL slide test technic with Span 60 in stead of cardiolipin in the original ethanol antigen in the concentration of 0.25 per cent. The content of cholesterol and lecithin is not a condition sine qua non but will increase the visibility of the flocculation. This test is called by us the APC test. The sera must be inactivated.

Of the 128 sera of blood donors which were tested 123 were negative, the rest of 5 sera showed a slight tendency to flocculate and were read as —?

Of the 62 sera sent from centres for maternal care (pregnant women) there were 55 negative, 3 positive and 4 —? cases.

Of the sera of patients in big central hospitals with various

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

departments there were 34 negative, 19 positive and 7 —? cases among the total of 60 cases tested.

The highest percentage of the positive results showed the municipal hospital in Helsinki where only internal or infectious cases are treated. Of 50 sera there were 24 positive, 21 negative and 5 —? results.

All the positive sera were titrated. Most of them were positive still in the dilution 1/4 or 1/8. The highest titres were 1/128 in one pneumonia and in one agranulocytosis case.

The positivity of the examined sera did not follow any other clinical symptom or any laboratory test performed. The most parallel feature was the sedimentation rate but there were many discrepancies. The cases with high titre were usually cases of diseases with tissue destruction.

The correlation of the new test with the C-reactive protein content of the sera is under study.

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## ESTIMATION OF C-REACTIVE PROTEIN BY A NEW METHOD

by

M. TUOMIOJA<sup>1</sup>, P. KAJANNE, O. WAGER<sup>1</sup>, and K. HÄLLSTRÖM

(Received for publication April 4, 1956.)

In a previous paper two of the present writers described a new flocculation test based on the principle of the VDRL slide test, well known in the serology of syphilis (1). The substance used as antigen was a technical product stated to contain a mixture of sorbitan monostearates. This new test, known as the APC test, was compared with the anti-C-reactive protein serum precipitation test. The antiserum was delivered by Messrs. Schieffelin & Co., New York, and the capillary method recommended in their prospect was used.

Fifty-five sera, first tested by the APC test, were submitted to one of us who did not know the results. The sera were tested inactivated in the APC test and active in the anti-serum test. The results are listed in the table below:

No. of Serum	APC	APC Titre in Dils	Anti-Serum Test
1	+	8	4 +
2	+	8	3 +
3	—?		(+)
4	—		—
5	+	16	5 +
6	—		—
7	—		—
8	+	16	5 +
9	+	8	4 +
10	+	8	4 +
11	+	4	2 +
12	+	8	4 +
13	+	16	2 +
14	+	4	3 +
15	+	8	4 +

<sup>1</sup> Aided by grants from the Sigrid Jusélius Foundation.

No. of Serum	APC	APC Titre in Dils	Anti-Serum Test
16	—		(+)
17	—		—
18	+	4	3 +
19	+	4	3 +
20	—		—
21	—		1 +
22	+	32	4 +
23	—		—
24	+	1	1 +
25	+	2	2 +
26	—		—
27	—		—
28	—		—
29	+	32	4 +
30	—		—
31	—		1 +
32	—		(+)
33	+	8	3 +
34	+	8	4 +
35	—		—
36	—		—
37	—		—
38	—		—
39	+	32	4 +
40	+	16	3 +
41	—		—
42	+	16	4 +
43	+	16	3 +
44	—		(+)
45	—		1 +
46	+	4	3 +
47	+	16	4 +
48	—		—
49	+	4	1 +
50	—		—
51	—		—
52	+	1	1 +
53	+	4	2 +
54	—?		3 +
55	+	16	3 +

Small differences in the results with weak positive sera are accounted for by the different sensitivities or by the gradation of reading. The only real discrepancy is to be found in the results obtained with serum No. 54. The APC test was repeated with this serum and was  $\pm$ ; in titration the reading was  $\pm$ , +, ++, +, —?, —. So there was a marked inhibition zone, as is usual in the case of active sera.

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## HISTAMINANTAGONISTISCHE WIRKSAMKEIT VON ANTI- HISTAMINICA UND ATROPIN AN DER MEERSCHWEINCHENHAUT

von

RIITTA TAKKUNEN und JOACHIM ALBERTY

(Bei der Schriftleitung eingegangen am 6. März 1956)

In vorausgegangenen Untersuchungen wurde gezeigt, dass Wirkungsstärke und Wirkungsspezifität von spezifischen Antihistaminica an Darm und Lunge des Meerschweinchens sehr verschieden sind (1, 2). An der isolierten durchströmten Lunge waren erheblich höhere Antihistamin-Konzentrationen notwendig, um die gleiche antagonistische Wirkung zu erzielen wie am isolierten Darm. Atropin wirkte dagegen als unspezifischer Histaminantagonist an beiden Organen annähernd gleich stark.

Durch entsprechende Untersuchungen an einer andersartigen Wirkungseigenschaft von Histamin sollten diese bisherigen Ergebnisse ergänzt und erweitert werden. Hierzu wurden Bestimmungen der antagonistischen Wirksamkeit von Antihistaminica und Atropin an der durch intracutan injiziertes Histamin hervorgerufenen vasculären Permeabilitätssteigerung beim Meerschweinchen durchgeführt.

### METHODE

Bei Meerschweinchen, welche eine intravenöse Injektion des kolloidalen Farbstoffs Trypanblau erhalten haben, tritt aus den durch Einwirkung nachfolgend intracutan injizierten Histamins erhöht permeablen Kapillaren gefärbtes Plasma in das subcutane Gewebe. Im Bereich der Injektionsquaddel entsteht dadurch ein annähernd kreisförmig begrenzter blauer Fleck. Bei konstanter Farbstoffdosis und gleichen intracutanen Injektions-

volumina hängen Farbintensität und Ausdehnung solcher in gleichen Hautbezirken hervorgerufener »Hautreaktionen« von der Grösse der Histamin-Konzentration ab (8).

Die Rückenhaut weisser Meerschweinchen von etwa 300 g Gewicht wurden enthaart mit einer mit Wasser anzurührenden Paste aus 10 Teilen Bariumsulfid, 1 Teil Seifenpulver, 7 Teilen Weizenstärke und 7 Teilen Talkum. In die unter kurzer leichter Äthernarkose freigelegte vena jugularis wurden 2 ml/kg einer 1%igen wässrigen Lösung von Trypanblau »Grübler« injiziert. Die Schnittwunde wurde geklammert und etwa 4—5 Minuten danach Histamin in verschiedenen Konzentrationen intracutan gegeben. Die Injektionen erfolgten in die Haut des Rückens zu beiden Seiten mit etwa 1 cm Abstand von der Mittellinie in einer Reihe, die von unteren Rand der Schulterblätter bis etwa zur Höhe der Kniegelenke des sitzenden Tieres lief. Einem Tier wurden durchschnittlich 8, maximal bis zu 12 intracutane Injektionen gegeben. Die Injektionsvolumina betrugen 0.05 ml. Die Injektionen wurden mit Ganzglas-Tuberkulinspritzen (Becton, Dickinson & Co., Rutherford N. J.) und 10 mm langen Injektionskanülen No. 35 (Luer) vorgenommen.

Die Hautreaktionen entwickelten sich innerhalb von 12—15 Minuten vollständig und wurden dann abgelesen. Es konnten 5 voneinander gut abgrenzbare *Intensitätsstufen* (»I.St.«) unterschieden werden, die mit den Ziffern 0—1—2—3—4 bezeichnet werden sollen:

- I. St. 4: Gleichmässig dunkelblauer kreisförmig begrenzter Fleck.
- I. St. 3: Dunkelblauer kreisförmig begrenzter Fleck mit vereinzelt leichten Aufhellungen.
- I. St. 2: Gleichmässig hellblauer kreisförmig begrenzter Fleck.
- I. St. 1: Schwache fleckige hellblaue Anfärbungen, keine deutliche Kreisform und keine deutliche Begrenzung gegenüber normaler Haut.
- I. St. 0: Kein Farbstoffaustritt im Injektionsbereich, gleiches Bild wie bei Injektion physiologischer Kochsalzlösung.

Strich- oder punktförmige, eindeutig von Gefässverletzungen durch die Injektionskanüle herrührende Blaufärbungen wurden nicht berücksichtigt.

Antagonistische Wirkungen wurden geprüft, indem die Antagonistensubstanz in bestimmten Konzentrationen gemeinsam mit Histamin im gleichen Injektionsvolumen intracutan appliziert wurde. In einer zweiten Versuchsreihe wurde der Antagonist etwa 30 Minuten vor Histamin intraperitoneal injiziert.

In den Versuchen mit gemeinsamer intracutaner Applikation von Histamin und Antagonisten wurde die Wirkung von mindestens 4 verschiedenen Konzentrationen jedes Antagonisten gegen wenigstens 2, in einzelnen Fällen 3 verschiedene Histamin-Konzentrationen geprüft. Geeignete Antagonisten-Konzentrationen wurden in Vorversuchen ermittelt. Die Wirkung jeder einzelnen Antagonisten-Konzentration gegen jede Histamin-Konzentration wurde in mindestens 10 Einzelinjektionen bestimmt, die in zufälliger Folge angeordnet waren und sich auf mehrere Tiere verteilten. Aus der Summe der Intensitätsstufenzahlen zusammen-

gehöriger Einzelbestimmungen wurde der Mittelwert gebildet. Da es sich dabei nicht um exakte Messwerte im strengen Sinne handelte, unterblieb eine weitere statistische Bearbeitung des Ergebnismaterials. Neben der Reaktionsintensität wurde von allen Reaktionen, soweit möglich, der Durchmesser gemessen.

Kontrollen der unbeeinflussten Histaminwirkung wurden an jedem Versuchstier vorgenommen. In der Versuchsreihe mit vorausgehender intraperitonealer Antagonisteninjektion dienten unbehandelte Tiere als Kontrollen.

#### Verwendete Pharmaka:

Histamin bihydrochlorid

Pyrilamin maleat (NNR) (Mepyramin maleat NFN)

Diphenhydramin hydrochlorid (NNR, NFN)

Antazolin methansulfonat (NNR) (Imidamin methansulfonat NFN)

Atropinsulfat

Die Injektionslösungen wurden am Versuchstag frisch mit physiologischer Kochsalzlösung zubereitet. Alle Konzentrations- und Dosisangaben beziehen sich auf die Salze.

### ERGEBNISSE

1. *Konzentrations-Wirkungs-Beziehungen von Histamin.* — Aus dieser Bestimmung gehen Bereich und Grössenunterschiede von Histamin-Konzentrationen abstufbarer Wirkung hervor. Die Ergebnisse sind in *Tabelle 1* aufgeführt. Die Zunahme der Wirkungsstärke verläuft zwischen  $10^{-6.5}$  und  $10^{-5}$  g/ml Histamin dem Konzentrationslogarithmus annähernd linear proportional (*Abbildung 1*). Mit Konzentrationsunterschieden von 1: 3,16 ( $10^{0.5}$ ) sind die Inten-

TABELLE 1

INTENSITÄT DER HAUTREAKTIONEN DURCH INTRACUTANE HISTAMININJEKTIONEN

0.05 ml Histamin- bihydrochl g/ml	Intensitätsstufen (*I.St.*) (Einzelwerte)	Mittel- wert
$10^{-6.5}$	1 1 0 0 0 0 0 0 0	0.25
$10^{-6}$	3 2 2 2 2 2 1 1 1 1 1 1 1 1 0	1.5
$10^{-5.5}$	4 3 3 3 3 3 3 3 3 2 2 2 2 2 2	2.7
$10^{-5}$	4 4 4 4 4 4 4 4 4 4 4 4 4 4 4	4
$10^{-4.5}$	4 4 4 4 4 4 4 4 4 4 4 4 4 4 4	4
$10^{-4}$	4 4 4 4 4 4 4 4 4 4 4 4 4 4 4	4

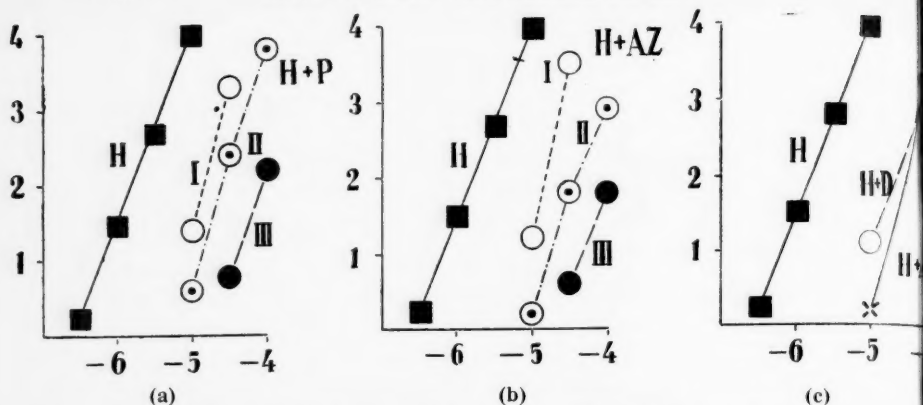


Abb. 1. — Konzentrations-Wirkungs-Beziehungen von Histamin ohne und mit Anwesenheit von Antagonisten (gemeinsame intracutane Injektion).

Abscisse: Log. (g/ml Histamin bihydrochlorid)

Ordinate: Intensitätsstufen der Hautreaktionen

H : Histamin allein

(a) H+P : Histamin + Pyrilamin mal. (I:  $10^{-6.5}$ , II:  $10^{-6}$ , III:  $10^{-5.5}$  g/ml)

(b) H+AZ : " + Antazolin meth. sulfon. (I:  $10^{-5.5}$ , II:  $10^{-5}$ , III:  $10^{-4.5}$  g/ml)

(c) H+D : " + Diphenhydramin hydrochl.  $10^{-5.5}$  g/ml

H+AT : " + Atropin sulf.  $10^{-4}$  g/ml

(Jeder Punkt der Kurven ist ein Mittelwert aus wenigstens 10 Einzelwerten)

sitätsunterschiede der Hautreaktionen gut ausgeprägt. Histamin  $10^{-5}$  g/ml wirkt gerade eben maximal, die Reaktionen auf Konzentrationen zwischen  $10^{-5}$  und  $10^{-4}$  g/ml zeigen keine deutlichen Intensitätsunterschiede. Bei Anwendung höherer Konzentrationen als  $10^{-4}$  g/ml wurde Hemmung des Farbstoffaustritts im zentralen Quaddelbereich beobachtet, die von Miles und Miles (8) ebenfalls beschrieben und auf anhaltende Arteriolenkontraktion durch hohe Histamin-Konzentrationen zurückgeführt wurde.

2. Wirkung bei gemeinsamer intracutaner Applikation von Histamin und Antagonist. — Die mit allen geprüften Histamin-Antihistamin-Kombinationen erhaltenen Intensitätsstufen-Mittelwerte sind in Tabelle II aufgeführt. Reduktion der maximalen permeabilitätssteigernden Histaminwirkung (I. St. 4) in den Bereich der Intensitätsstufe 1 und darunter bedeutet fast vollständigen Antagonismus. Auf der Basis dieses antagonistischen Effekts ergibt sich die relative Wirkungsstärke der Antagonisten Pyrilamin : Diphenhydramin : Antazolin : Atropin mit 100 : 32 : 10 : 1 in der Versuchs-

TABELLE 2

INTENSITÄT DER HAUTREAKTIONEN BEI GEMEINSAMER INTRACUTANER INJEKTION  
VON HISTAMIN UND HISTAMINANTAGONISTEN  
(Intensitätsstufen-Mittelwerte aus je 10 Einzelbestimmungen)

Histamin bihydrochl. g/ml	Ø	mit Antazolin m. sulfon. Log. (g/ml)					mit Atropin sulf. Log. (g/ml)				
		-7	-6.5	-6	-5.5	-5	-6.5	-6	-5.5	-5	-4.5
$10^{-5}$	4	3.2	1.4	0.6			3.6	2.3	1.1		
$10^{-4.5}$	4		3.3	2.4	0.8						
$10^{-4}$	4			3.8	2.2	1.3			3.3	1.7	0.9

Histamin bihydrochl. g/ml	Ø	mit Pyrilamin mal. Log. (g/ml)					mit Diphenhydr. hydrochl. Log. (g/ml)				
		-6	-5.5	-5	-4.5	-4	-5	-4.5	-4	-3.5	-3
$10^{-5}$	4	3.2	1.2	0.2			2.7	1.5	0.2		
$10^{-4.5}$	4		3.5	1.8	0.6						
$10^{-4}$	4			2.9	1.8	1.0			3.4	2.6	0.9

serie mit  $10^{-4}$  g/ml Histamin, und  $100:32:>10$ ,  $<32:>1$ ,  $<3,2$  in der Versuchsserie mit  $10^{-5}$  g/ml Histamin, also annähernd die gleichen Wirksamkeitsverhältnisse in beiden Versuchsreihen. Auch in der Versuchsreihe mit  $10^{-4.5}$  g/ml Histamin wirkt Pyrilamin 10 mal stärker als Antazolin. Atropin zeigt auch an diesem Testobjekt eindeutige histaminantagonistische Wirkungen, wenn auch in relativ hohen Konzentrationen.

Aus Tabelle II geht weiter hervor, dass in den geprüften Konzentrationsbereichen der Effekt einer Histamin-Antihistamin-Kombination annähernd gleich bleibt, wenn die Konzentration beider Komponenten um den gleichen Faktor gesteigert wird.

3. Wirkung des Antagonisten bei vorausgehender intraperitonealer Injektion. — Um festzustellen, ob die zeitlichen Bedingungen und der Weg der Zufuhr des Antagonisten für dessen Wirksamkeit von wesentlicher Bedeutung waren, wurden Versuche mit vorausgehender intraperitonealer Applikation von Pyrilamin durchgeführt. Die Ergebnisse sind in Abbildung 2 dargestellt. Die verabreichten Dosen von 1 und 3.16 mg/kg intraperitoneal ( $10^{-6}$  und  $10^{-5.5}$  g



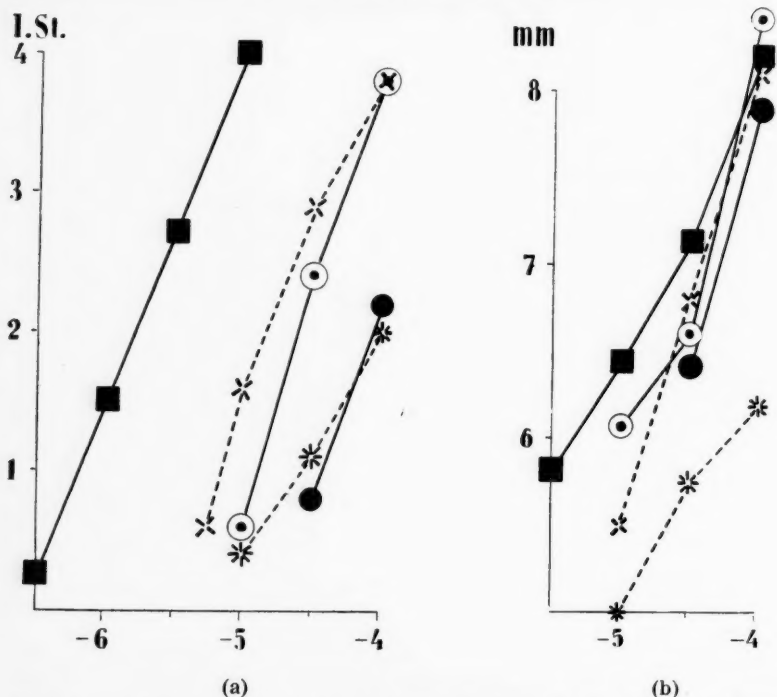


Abb. 2. — Wirkung von Pyrilamin bei intraperitonealer oder subcutaner Anwendung auf die Intensität (a) und den Durchmesser (b) der histaminbedingten Hautreaktionen.

Abscissen: Log. (g/ml Histamin bihydrochl.)

Ordinaten: (a) Intensitätsstufen, (b) Durchmesser (mm)

- —: Histamin alleine,
- × —: nach Pyrilamin 1 mg/kg = 10<sup>-6</sup> g/g Tier intraperitoneal,
- \* —: „ „ 3.16 „ = 10<sup>-5.5</sup> „ „
- —: mit „ 10<sup>-6</sup> g/ml intracutan,
- —: „ „ 10<sup>-5.5</sup> „ „

(Hautreaktionen nach 3.16 mg/kg Pyrilamin intraperitoneal, deren Durchmesser nicht messbar waren (I. St.-Bereich 1 und darunter), wurden bei der Berechnung der Durchmesser-Mittelwerte nicht berücksichtigt. Die übrigen Punkte aller Kurven sind Mittelwerte aus wenigstens je 10 Einzelwerten).

Pyrilamin pro g Tier) bezogen sich auf die bei gemeinsamer intracutaner Injektion geprüften Konzentrationen 10<sup>-6</sup> und 10<sup>-5.5</sup> g/ml Pyrilamin, welche bei dieser Applikationsweise die Wirkung von 10<sup>-5</sup> beziehungsweise 10<sup>-4.5</sup> g/ml Histamin fast aufhoben (vgl. Tabelle II). Aus Abbildung 2a geht hervor, dass die histaminantagonistische Wirkung der vorausgehend intraperitoneal ver-

abreichen, auf »Gewebs-Konzentration« bezogenen Dosen annähernd ebenso stark ist wie die Wirkung der entsprechenden gemeinsam mit Histamin intracutan injizierten Konzentrationen.

Während intraperitoneal und intracutan verabreichtes Pyrilamin die *Intensität* der Permeabilitätssteigerung annähernd gleich stark hemmt, wird ihre *Ausdehnung* dagegen durch intracutan gemeinsam mit Histamin injiziertes Pyrilamin in den verwendeten Konzentrationen nur wenig, durch vorausgehend intraperitoneal gegebenes Pyrilamin in den entsprechenden Dosen dagegen deutlich stärker beeinflusst (*Abbildung 2b*).

#### DISKUSSION

*Methode.* — In ihren eingehenden Untersuchungen über vasculäre Reaktionen in der Meerschweinchenhaut durch Histamin und histamin-freisetzende Substanzen bestimmten Miles und Miles (8) die Intensität der Hautreaktionen mittels des *Durchmessers* der Blaufärbung. Diese Methode bietet den Vorteil der statistischen Auswertbarkeit der Ergebnisse und ist subjektiven Fehlermöglichkeiten weniger stark unterworfen als die Bewertung der Farbintensität. Sie schien jedoch für unsere Untersuchungen weniger geeignet: Bei schwachen Reaktionen im Intensitätsstufen-Bereich 1 und darunter wird die Grenze zwischen gefärbter und normaler Haut unscharf, eine deutliche kreisförmige Begrenzung ist häufig überhaupt nicht mehr vorhanden und sichere Messwerte für den Reaktions-Durchmesser sind dann nicht mehr zu bestimmen. Andererseits wird durch die gemeinsam mit Histamin intracutan injizierten Antagonisten die Grösse des Reaktionsfeldes noch wenig beeinflusst, wenn die Hemmung der Permeabilitätssteigerung schon eindeutig an der verringerten Farbintensität erkennbar ist. Bei intraperitonealer Verabreichung von Antagonisten wurde zwar Farbintensität und Reaktions-Durchmesser deutlich beeinflusst. Unserer experimentellen Zielsetzung entsprechend mussten die Wirk-samkeitsbestimmungen der Antagonisten jedoch einheitlich mit ihrer intracutanen Anwendung durchgeführt werden, da mit schwächer wirksamen Substanzen wie Antazolin und Atropin nur auf diese Weise genügend hohe Konzentrationen an das Reaktionssubstrat herangeführt werden konnten.

*Wirkung von Pyrilamin bei verschiedener Applikationsweise.* — Unter der Annahme, dass Pyrilamin etwa 40 Minuten nach seiner intraperitonealen Injektion annähernd gleichmässig im Organismus verteilt ist, kann die injizierte Dosis als Gewebskonzentration angegeben und in grössenordnungsmässiger Annäherung mit der lokalen Wirkung der entsprechenden intracutan verabreichten Konzentration verglichen werden. Dabei dürfte die nach peritonealer Resorption erreichte theoretische Konzentration in der Haut eher zu hoch veranschlagt sein, sofern keine spezifische Affinität für

Pyrilamin besteht. Der Vergleich der Versuchsergebnisse mit intraperitoneal und intracutan verabreichtem Pyrilamin bestätigt diese Vorstellungen (Abbildung 2a).

Es spielt für die antagonistische Wirkungsstärke offenbar keine wesentliche Rolle, ob Pyrilamin gleichzeitig mit Histamin an das Substrat gelangt oder vorher auf dem Weg über den allgemeinen Kreislauf herangeführt wurde und zum Zeitpunkt der Histamin-Applikation bereits anwesend ist. Demgegenüber bedarf es in der Versuchsanordnung am isolierten Meer-schweinchendarm bis zur vollen Entwicklung des histaminantagonistischen Effekts einer Kontaktzeit von Pyrilamin und anderen Antihistaminica mit dem Organ von etwa 7—10 Minuten (1, 9). Eine Erklärung dieser Differenz dürfte am ehesten in einem verschiedenen Verhältnis der Adsorptionsgeschwindigkeiten von Antihistamin/Histamin an unterschiedlichen spezifischen Zellrezeptoren zu suchen sein.

Dem relativ geringen Einfluss intracutan gemeinsam mit Histamin injizierten Pyrilamins auf die Reaktionsausdehnung könnten Unterschiede in der Gewebsaffinität beider Substanzen zugrundeliegen. Wenn intracutan injiziertes Pyrilamin stärker von subcutanen Gewebe adsorbiert wird als Histamin, welches sich leicht mit der Injektionsflüssigkeit ausbreitet (8), so ist der Konzentrationsabfall vom Zentrum zur Peripherie der Injektionsquaddel für Pyrilamin steiler als für Histamin, und die antagonistische Wirksamkeit nimmt peripherwärts ab. Nach peritonealer Resorption ist die Gewebskonzentration von Pyrilamin dagegen gleichmässig und nur die Konzentration intracutan injizierten Histamins sinkt vom Quaddelzentrum zur Peripherie, so dass der antagonistische Effekt peripherwärts zunimmt.

*Konzentrations-Wirkungs-Beziehungen.* — Die Konzentrations-logarithmus-Wirkungs-Kurven von Histamin ohne und mit Anwesenheit von intraperitoneal oder intracutan zugeführten Antagonisten sind annähernd parallel (*Abbildung 1 u. 2*). Bei Einwirkung einer bestimmten Antagonisten-Konzentration steigt also die für eine bestimmte (nicht-maximale) Wirkung erforderliche Histamin-Konzentration um einen bestimmten Faktor, oder anders ausgedrückt: Durch Einwirkung einer bestimmten Antagonisten-Konzentration wird die Empfindlichkeit des Testorgans gegen Histamin um einen bestimmten konstanten Faktor gesenkt. Dieser »Schutzfaktor« (7, 11) *F* ist vom Grad der Histaminwirkung unabhängig und kennzeichnet die absolute Wirkungsstärke der anwesenden Antagonisten-Konzentration. Er ergibt sich aus dem horizontalen Abstand der Konzentrations-Wirkungskurven von Histamin ohne und mit Anwesenheit des Antagonisten (Verhältnis der

mit und ohne Anwesenheit des Antagonisten gleichwirksamen Histamin-Konzentrationen).

Die antagonistische Wirkung der Antihistaminica und von Atropin an der histaminbedingten Permeabilitätssteigerung in der Haut folgt anscheinend in ihren Grundzügen den gleichen formalen Gesetzen (4, 5) wie am Blutdruck (7, 10), am isolierten Darm (1, 9) und an der isolierten Lunge (2). Dadurch wird ein quantitativer Vergleich der an diesen Testobjekten bestimmten Wirksamkeitswerte untereinander möglich (siehe unten).

Das Verhältnis wirkungsgleicher Konzentrationspaare von Histamin liegt ebenfalls der von Schild (9) vorgeschlagenen Bezeichnung der Antihistamin-Wirksamkeit  $pA_x$  zugrunde. ( $pA_x$  ist der negative Logarithmus der molaren Antihistamin-Konzentration, die den Effekt der x-fachen (= F-fachen) Histamin-Konzentration auf den Effekt der einfachen Histamin-Konzentration reduziert).

Der prozentuale Anteil der gegebenen Histamin-Konzentration, deren Wirkung durch eine bestimmte Antagonisten-Konzentration (-Dosis) aufgehoben wird, ergibt sich aus  $100 \left(1 - \frac{1}{F}\right)$  und ist gleichfalls zur Charakterisierung und Veranschaulichung der Antagonisten-Wirkungsstärke geeignet (1, 2).

*Histaminantagonistische Wirksamkeit an der Haut und anderen Organen.* — In Tabelle III a und b sind diejenigen Konzentrationen von Antihistaminica und Atropin zusammengestellt, welche im Versuch am isolierten Darm (1, 9), an der isolierten durchströmten Lunge (2) und an der Haut des Meerschweinchens die Wirkung von 50 beziehungsweise 90% der gegebenen Histamin-Konzentration aufheben ( $F = 2$  beziehungsweise 10). Die Lücke für den Pyrilaminwert an der isolierten Lunge wird durch Angabe des chemisch und in seiner Wirksamkeit Pyrilamin nahestehenden Antihistamins Tripelennamin überbrückt.

Aus dem Vergleich geht hervor, dass Atropin als unspezifischer Histaminantagonist an den verschiedenen Testobjekten weitgehend gleich stark wirkt. Ebenso liegen auch die an der isolierten Lunge und an der Haut gleichwirksamen Konzentrationen von Antihistaminica im gleichen Grössenbereich.

Demgegenüber zeigen sich zwischen den an der Haut und am isolierten Darm gleichwirksamen Konzentrationen desselben Antihistamins beträchtliche Differenzen. Der Unterschied ist am grössten für das stärkste der Antihistamine, Pyrilamin, welches an der Haut erst in 300—700 mal höheren Konzentrationen ebenso stark

TABELLE 3

AN VERSCHIEDENEN TESTOBJEKTEN GLEICHWIRKSAME KONZENTRATIONEN VON HISTAMINANTAGONISTEN

(Konzentrationsangaben in g/ml der Salze)

(Die in Klammern gesetzten Zahlen beziehen sich auf den Literaturnachweis)

Antagonist	Aufhebung der Wirkung von 50 % der gegebenen Histamin-Konzentration ( $F = 2$ ) an		
	isol. Meersch.-darm (1.9)	isol. Meersch.-lunge (2)	Haut des Meersch.
Pyrilamin mal. ....	10-9.87		10-7
Tripelennamin hydrochl. ....	10-9.2	10-7.7	
Diphenhydramin hydrochl. ....	10-8.4		> 10-6.5, < 10-6
Antazolin meth. sulfon. ....	10-8	> 10-7.3, < 10-6.6	10-6
Atropin sulf. ....	10-5.8	10-5.12	> 10-5, < 10-4.5
	Aufhebung der Wirkung von 90 % der gegebenen Histamin-Konzentration ( $F = 10$ )		
Pyrilamin. mal. ....	10-8.77		10-6.5
Tripelennamin hydrochl. ....	10-8.21	> 10-6.7, < 10-6	
Diphenhydramin hydrochl. ....	10-7.4		> 10-6, < 10-5.5
Antazolin meth. sulfon. ....	10-6.92	10-5.7	> 10-6, < 10-5.5
Atropin sulf. ....	10-4.62	> 10-4.12, < 10-4	10-4.5

wie am Darm wirkt, während Antazolin als relativ schwächstes der Antihistamine an der Haut etwa 50—100 mal höhere Konzentrationen erfordert. Relative Wirksamkeitsunterschiede zwischen den verschiedenen Antihistaminsubstanzen sind dadurch am Hauttest wesentlich geringer als im Versuch am isolierten Darm. Ebenso verringert sich die durch das Verhältnis von Antihistamin- zu Atropin-Wirksamkeit gekennzeichnete histaminantagonistische Wirkungsspezifität, für Pyrilamin von 4 Zehnerpotenzen am isolierten Darm auf 2 Zehnerpotenzen an der Haut, für Antazolin entsprechend von 2.3 auf etwas über 1 Zehnerpotenz. Pyrilamin wirkt an der Haut nur etwa ebenso spezifisch histaminantagonistisch wie das schwächste der spezifischen Antihistamine Antazolin am isolierten Darm.

Diese Ergebnisse bestätigen die bereits an anderer Stelle getroffene Feststellung (1, 2), dass der Begriff der Wirksamkeit und Wirkungsspezifität von Antihistaminica, soweit er sich überwiegend auf Größenordnungen wirksamer Konzentrationen gründet, unvollständig ist. Er bedarf der Angabe des Angriffspunktes.

In dem an diesen Versuchsobjekten durchgeführten Vergleich stellt sich die bemerkenswert hohe Wirksamkeit und Wirkungsspezifität von Antihistaminica am isolierten Meerschweinchendarm als ein relativ allein stehendes Phänomen dar. Diesem Testobjekt kommt daher für bestimmte Fragestellungen besondere Bedeutung zu. Als Grundlage einer allgemeineren, mehr von praktischen Gesichtspunkten der therapeutischen Anwendung ausgehenden Bewertung von Antihistaminsubstanzen dürften Ergebnisse von Wirksamkeitsbestimmungen am isolierten Meerschweinchendarm jedoch nur von begrenztem Wert sein.

Eine Erklärung dieser Befunde kann sich nur auf Vermutungen gründen. Quantitative Wirkungsunterschiede spezifischer Antihistaminica am isolierten Darm und an der Haut werden durch die Annahme verständlich, dass die Angriffspunkte von Histamin, dass heisst seine Eingriffe in Teilprozesse des Zellstoffwechsels und der Zellerregung, an der glatten Muskulatur und an der Kapillarschleimhaut verschieden sind. Dann spräche die quantitative Übereinstimmung der histaminantagonistischen Atropinwirkung an unterschiedlichen Wirkungsqualitäten von Histamin für einen gleichartigen, also nicht für die Qualität der Histaminwirkung spezifischen Angriffspunkt dieser Atropinwirkung in verschiedenen Zellsystemen. Der zunächst nur relativ auf Konzentrations-Größenordnungen bezogene Begriff der Unspezifität des Atropin/Histamin-Antagonismus wäre damit auch ursächlich begründet.

Sofern die Histaminwirkung an der Lunge überwiegend als Spasmus der glatten Bronchialmuskulatur gedeutet wird, ist es schwer verständlich, warum spezifische Antihistamine an diesem Testobjekt wesentlich schwächer wirken als an der glatten Muskulatur des Darmes. An einem Versuchspräparat, welches das Verhalten der glatten Bronchialmuskulatur allein wiedergibt, der isolierten Bronchialkette (3) aus Bronchialringen des Menschen, fanden Hawkins und Schild (6) gleiche histaminantagonistische Wirkungsstärke von Pyrilamin wie am isolierten Meerschweinchendarm. Die Beobachtung, dass Pyrilamin ebenso wie Antazolin die Histaminwirkung an der isolierten durchströmten Lunge dagegen nur etwa ebenso stark hemmt wie die histaminbedingte vasculäre



Permeabilitätssteigerung in der Haut, führt daher zu der Vermutung, dass die histaminbedingte Bronchostenose im ganzen Organ überwiegend als Folge der permeabilitätssteigernden Wirkungseigenschaft von Histamin zustandekommt.

#### ZUSAMMENFASSUNG

An der durch intracutan gegebenes Histamin hervorgerufenen Hautreaktion beim Meerschweinchen, welcher eine Permeabilitätssteigerung der Hautkapillaren zugrunde liegt, wurde die antagonistische Wirksamkeit von Pyrilamin, Diphenhydramin, Antazolin und Atropin bestimmt. Die Befunde wurden mit Ergebnissen von Wirksamkeitsbestimmungen am isolierten Darm und der isolierten Lunge verglichen.

Atropin wirkt an der Haut ebenso stark histaminantagonistisch wie am Darm und an der Lunge des Meerschweinchens. Mit der Annahme, dass Atropin an Rezeptoren angreift, welche für die Wirkungsqualität von Histamin nicht spezifisch sind, kann der Begriff der Unspezifität des Atropin-Histamin-Antagonismus ursächlich begründet werden.

Spezifische Antihistaminica wirken an der Haut wesentlich schwächer und auch relativ weniger spezifisch als am isolierten Meerschweinchendarm. Es wird angenommen, dass diese quantitativen Unterschiede darauf beruhen, dass die Angriffspunkte, an denen sich der Histamin-Antihistamin-Antagonismus abspielt, an der glatten Muskulatur des Darmes und an der Kapillarwand verschiedenartig sind.

Auf Grund der weitgehend übereinstimmenden Wirksamkeit spezifischer Antihistamine an der Lunge und an der Haut wird vermutet, dass die histaminbedingte Bronchostenose im ganzen Organ überwiegend als Folge der permeabilitätssteigernden Wirkungseigenschaft von Histamin zustande kommt.

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## PRACTICAL BLOOD VOLUME MEASUREMENTS WITH RADIOACTIVE CHROMIC CHLORIDE AND IODINATED HUMAN SERUM ALBUMIN

by

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A number of different methods are now available for measurement of the blood volume, and most of them possess an accuracy which is adequate for clinical use. The older methods include Sjöstrand's carbon monoxide method, which is chiefly used in Scandinavia, whereas the Evans' blue method has gained a wide application in the United States. Radioactive substances that have been used with advantage are radioactive phosphorus  $P^{32}$  (18, 16, 1, 20, 19, 4, 22, 3) and radioactive iron  $Fe^{59}$  (14, 5, 15, 10). Radioactive iodine  $I^{131}$  bound to albumin has also been employed (7, 6, 24, 2, 3, 9, 21).

Radioactive chromium  $Cr^{51}$  has been used in determination of both the circulating red cell mass and the plasma volume, for the latter purpose in the form of chromic chloride and for the former as sodium chromate. The former is suited for determinations of the plasma volume and the latter for measurements of the red cell mass (12, 13, 23, 8, 11).

The use of radioactive phosphorus has the drawback that the blood sample must be incubated with the active solution.

Furthermore, there is no accurate knowledge of the amount of phosphorus that becomes bound to the red cells and the plasma. Measurement of the samples is rather difficult, since phosphorus is

a pure beta radiator (beta ray energy 1.69 mev) and the measurement is carried out with insensitive Geiger tubes.

The use of iron presents some difficulty in practice since a special active red cell donator must be employed. The human or animal donator is given an intravenous injection of a solution containing radioactive iron, only after which labelled red cells are formed in the bone marrow.

Radioactive iodine has not been used very widely so far, because of the laborious preparation of iodinated serum albumin. Quite recently, however, the Radiochemical Centre, in England, has undertaken the preparation of iodinated human serum albumin.

In relative measurements the technique of employment of radioactive chromium has proved simple and rapid (8). Since the chromium can be used in two different forms, the simultaneous, independent determination of both plasma volume and red cell mass should be possible (11). Cationic chromium is bound immediately by the plasma proteins and it is possible to take the first sample already about 6 minutes after injection of the tracer dose. Following intravenous injection, 98 per cent of the chromium ions are bound to the proteins and the chromium content of the blood declines at the rate of the general protein turnover. The doses of chromium can be repeated on the same individual, thus making it possible to determine the blood volume after transfusion or haemorrhage. The tracer doses are so small (about 10  $\mu$ c) that there is no danger of radiation even after several doses. The half-life of radioactive chromium is 27 days and it is a gamma ray emitter and thus is suitable for measurement with a sensitive scintillation counter. The amount of chromium in each tracer dose is insignificantly low.

Radioactive iodine  $I^{131}$  HSA bound to human albumin has been available since June 1955 from the Radiochemical Centre in sterile rubber-stoppered vials. Evidently the demand for this substance for blood volume and other determinations has made mass preparation necessary. The serviceability of the product has been confirmed in very complete tests (9). It was found that the behaviour of  $I^{131}$  HSA in the circulation corresponds to that of normal plasma proteins, thus giving a highly reliable value for the plasma volume. The half-life of radioactive iodine is 8 days and it gives off both beta and gamma rays. Because of the gamma irradiation, measure-

ment is possible with a scintillation counter. The dosage for each determination is about  $10\ \mu\text{c}$ , and the amount of iodine is insignificant.

#### METHOD

The radioactive chromium was employed in the form of unsterilized chromic chloride prepared by the Radiochemical Centre, England, and the sterilization was carried out at this labo-

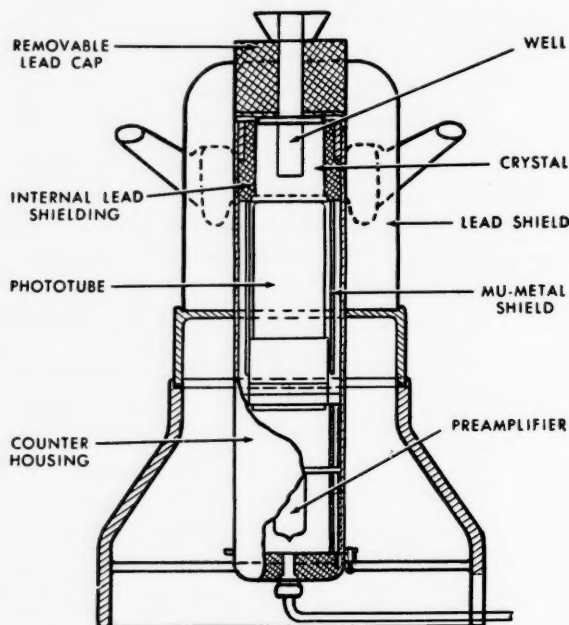


Fig. 1. — Well scintillation counter.

ratory. The chromium solution contained 5.6 mc in a volume of 20 ml. Samples of it were diluted so that the volume of 5–10 ml injected contained about  $10\ \mu\text{c}$ . Ten minutes after the injection of the active test dose the first blood sample of 10 ml was taken from the cubital vein in the opposite hand without stasis. Three to five samples were taken into heparinised centrifuge tubes. The samples were centrifuged for 30 minutes, and the haematocrit was determined. Four ml of plasma were separated from each sample. A dilution 1/50 was prepared from the chromium solution given to the subject. The plasma samples were then measured with a so-called

well scintillation counter tube — a scintillation counter equipped with a crystal having a hole — the construction of which is shown in the illustration.

The most important part in the scintillation counter is a cylindrical crystal of sodium iodine, about 5 cm in height and 4.5 cm in diameter, which is activated with thallium. The «well» is 1.7 cm in diameter and 3.8 cm deep. With the exception of its undersurface, the crystal is protected with a thin layer of aluminium. The undersurface of the crystal is attached to a light-sensitised photocathode in a photomultiplier tube. The surface of the photocathode is also treated with a thin layer of fluid which is transparent to light. Below the photomultiplier tube there is a preamplifier. All the above mentioned parts are placed in a metal cylinder, which in turn is placed in a rack whose upper part consists of a thick lead layer to minimise background radiation.

The operation of the scintillation counter may be briefly described as follows. As the radioactive substance in a test tube placed in the hole in the crystal emits gamma rays, the gamma quanta produce scintillations of light in the crystal. The scintillations hit the sensitised layer in the photomultiplier tube and release electrons from this layer, thus giving rise to electrical impulses. The impulses are first amplified in the photomultiplier tube itself and then in the preamplifier. From the preamplifier the pulses are conducted to the electronic counter. The scintillation counter equipped with a well crystal is about 750 times as sensitive as the Geiger tube for the measurement of gamma ray isotopes and it is capable of measuring samples up to a magnitude of  $10^{-5}$   $\mu$ c. In medical application this makes the use of very minute tracer doses possible.

The values obtained were noted on semi-logarithm paper. A straight line was drawn through the observed points and the initial value of the dilution was extrapolated to zero time as shown in Fig. 2.

The plasma volume and the total blood volume were calculated by the formulas:

$$P.V. = \frac{0.98 \cdot \text{Counts in tracer dose}}{\text{Counts of plasma samples at zero time}}$$

$$T.B.V. = \frac{\text{Counts in tracer dose}}{\text{Counts of native blood samples at zero time}}$$

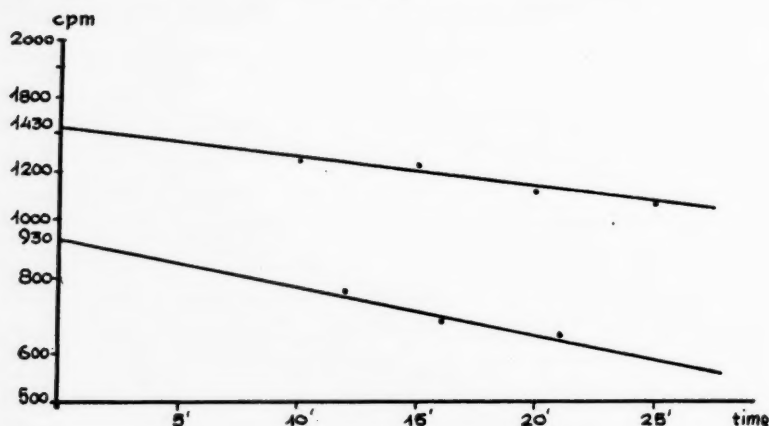


Fig. 2. — Determination of the initial counts on semi-logarithm paper from the decrease in the concentration of plasma (upper curve) and native blood (lower curve).

Example. — Patient X; body weight, 75 kg; height, 175 cm. Dose, abt.  $10 \mu\text{C Cr}^{51}\text{Cl}_3$  in 10 ml into the cubital vein. Plasma and native blood samples were taken from the cubital vein of the other arm and  $\text{Cr}^{51}\text{Cl}_3$  was diluted 1/50. Haematocrit value was 39 per cent.

Plasma Sample, 2 ml	Native Blood Sample, 2 ml	Scintillation Counts per Min.	
		Plasma	Native Blood
10 min.	12 min.	1260	747
15 "	16 "	1204	682
20 "	21 "	1139	639
25 "		1075	

Two ml of the tracer diluted 1/50 gave 9784 counts per min. Calculated from Fig. 2, the initial values were thus 1430 counts and 930 counts per min., respectively.

$$\text{P.V.} = \frac{0.98 \cdot 50 \cdot 9784}{1430} = 3418 \text{ ml, total blood volume} = 5600$$

$$\text{T.B.V.} = \frac{50 \cdot 10 \cdot 9784}{930} = 5260 \text{ ml.}$$

Difference between total volumes measured from plasma and native blood samples = 340 ml, equivalent to 6 per cent of the value measured from plasma. This difference is at least partly explainable by trapped plasma in the haematocrit determination.

## RESULTS

*Group I: Radioactive Chromium.* — To study the serviceability and accuracy of this new method we performed 22 blood volume determinations with radioactive chromic chloride on 11 patients with cancer. The measurements were at first made with a simple counter and repeated determinations were made after the transfusion of 800 ml of blood to each patient. Such marked fluctuations were seen in the measured plasma volumes that the accuracy of the method seemed doubtful. A new series of tests was therefore performed after the Wihuri Research Institute was able to procure a more sensitive counter. The results of the measurements in the second series are shown in Table 1.

TABLE 1

Subject No.	Measurement from Native Blood=N Plasma = P	Sex	Weight/Height	Hct	P.V. ml	P.V. ml/kg
			kg/cm			
5	P	♂	82/175	40	3440	42
6	P	♂	57/168	35	1940	34
7	{N P	♂	57/170	34	{4390 4390	{77 77
8	N	♀	43/154	43	2020	47
9	N	♂	55/174	35	1550	28
10	P	♂	72/167	40	3600	50
11	{N P	♂	75/175	39	{3150 3370	{42 45

Subjects No. 5, 7, 9, 10 and 11 had pulmonary carcinoma and subjects 6 and 8 oesophageal carcinoma. Lobectomy and thoracoplasty had been performed in case 9.

Simultaneous measurements were made from plasma and native blood on patients 7 and 11; both procedures gave uniform results. On the other hand, parallel determinations did not in all cases give similar values for the plasma volume and a tendency to excessively high values was observed. This was probably due to an irregular resorption of the test substance in some tissues. The fall in the concentration curve, it is true, had a similar slope in all the patients. The excessive plasma or blood volume values might



also be attributed to perivenous injection. However, minute attention was paid by us to this possibility and we feel that this could not be the reason here.

*Group II: Radioactive Iodinated Albumin.* — Blood volume determinations with radioactive iodinated albumin were made on 12 patients from native blood. The method employed was the same used in the determinations with chromium and the subjects were patients with pulmonary carcinoma who were in a good physical condition. The results are shown in Table 2.

TABLE 2

Subject No.	Measurement from Native Blood = N Plasma = P	Sex	Weight/Height	Hct	P.V. ml	P.V. ml/kg
			kg/cm			
12	N	♂	57/159	40	2000	35
13	N	♂	66/178	41	3040	46
14	N	♂	64/162	45	2750	43
15	N	♂	65/165	40	2540	39
16	N	♂	68/180	35	3550	52
17	N	♂	52/165	31	3060	59
18	N	♂	67/170	51	2340	35
19	N	♂	64/163	43	3000	47
20	N	♂	56/173	38	3190	57
21	N	♂	54/168	33	3080	57
22	N	♂	94/171	41	3380	36
23	N	♂	59/172	38	3250	55

The plasma volumes obtained with  $I^{131}$  HSA were fully in agreement with the probable values. The mean total blood volume (73.9 ml/kg or 2685 ml/sq.m.) agrees fully with the normal values reported in the literature. In an earlier study, also, one of us (17) found that the blood volume of patients with pulmonary carcinoma is the same as that of normal persons. There were no unexpected or inexplicable drawbacks in the blood volume determinations. The technique of determination was very simple, since with the aid of a good well-type crystal the measurements can be carried out from non-centrifuged native blood. The concentration curve was similar for all the patients and very gradual, which facilitates extrapolation. The only drawback that might be mentioned is the short half-life of the substance.

## SUMMARY

A brief review is given of the methods previously employed for the determination of plasma volume and red cell mass with radioactive substances. Plasma volume determinations were performed with radioactive chromic chloride and iodinated human serum albumin (IHSA) on patients with pulmonary and oesophageal carcinoma. The measurements with chromium were made from plasma and non-centrifuged native blood. The values obtained were of the same order of magnitude as those given by well-known methods previously employed. For a number of patients, however, the values measured with chromium chloride seemed to be too high and there was marked deviation in the values obtained in duplicate determinations. For this reason the accuracy of the method appears doubtful.

The determinations with IHSA were made from non-centrifuged native blood, which makes the method a rapid one and suitable also for clinical use. The observed values were in agreement with the blood volumes obtained by other methods. Regardless of its short half-life,  $I^{131}$ HSA is considered by the authors well applicable for clinical use, especially if the counter tube is sufficiently sensitive to permit determinations from native blood without preceding centrifugation.

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## INDUCTIVE ACTION OF NORMAL AND LEUCEMIC BONE-MARROW OF THE RAT

EXPERIMENTS WITH AMPHIBIAN EMBRYOS<sup>1</sup>

by

LAURI SAXÉN and SULO TOIVONEN

(Received for publication April 5, 1956)

The similarities and dissimilarities between malignant growth and embryonic induction have often been discussed (5, 7, 10). However, in only a few cases has the technique of developmental mechanics been applied to the study of this question. Using this technique, Woerdeman (21) used as inductors malignant tissues (Walker Rat Carcinoma, Peyton-Rous Sarcoma) and thus obtained neural inductions. According to the usual technique of that time the implants were fresh and the culture period of the test larvae very short — factors which, in our view, lessen the significance of the results. In addition to this older result, we have at our disposal some further results which, according to several authors, link the problems of malignant growth and embryonic induction. Various authors have shown that certain hydrocarbons known to have carcinogenic effects have a definite inductive action when implanted in young gastrulae; as a result the host animal develops secondary neural structures (12, 19, 20).

When trying to investigate the differences which may possibly exist in the inducing power of normal and malignant tissue, one has to choose a tissue as homogeneous and pure as possible. In addition, the inducing effect of the normal tissue should be strong

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<sup>1</sup> This investigation was supported by a grant from President J. K. Paasikivi's Fund for Cancer Research.

and readily analyzable. For this purpose we have chosen the bone-marrow of normal and leucemic rats. In our earlier investigations (14, 15, 17, 18) we have established that the bone-marrow of the guinea-pig is a strong and nearly pure mesodermal inductor. These inductions are, in the present view, caused by a specifically acting inducing agent, which is probably protein in nature (18, 22). The aim of the following investigation is to find out whether any changes in the mesoderm-inducing action of the bone-marrow are manifested when the tissue becomes malignant.

#### MATERIAL AND METHODS

As implant material bone-marrow tissue taken from the femur of a normal and a leucemic rat was used. The inductive action of the bone-marrow of the rat had not been studied earlier. Since a corresponding leucemic strain was not available in the guinea-pig, we were not able to experiment with the bone-marrow of the guinea-pig, the inductive action of which is well known from earlier investigations. The leucemic rat had received a transplant of a »transplantable myeloid rat leukemia» (developed by Dr. H. Shay, Fels Research Institute, Temple University, Philadelphia and given to us by Prof. H. Teir, Pathological Laboratory, University of Helsinki). The blood count of the rat before sacrifice was the following:

Leucocytes 52.000  
Segmented neutrophils 49 per cent  
Myelocytes 10 per cent  
Lymphocytes 41 per cent  
Normoblasts 35/100 Leuc.

The healthy control rat was of the same age and strain.

The rats were killed, and the femurs removed and cleaned; the bone-marrow was then dissected out and fixed in 70 per cent alcohol. The fixed bone-marrow was preserved in the same alcohol and the implantations were performed within 24—72 hours.

The implantation technique of Mangold was used. The test larvae (*Triturus vulgaris*) were cultured for about 14 days in Holtfreter-Ringer, fixed, serially sectioned and examined microscopically. Especially it will be emphasized that the preparation and later treatment of the bone-marrow, as well as the operations, were performed in exactly the same way in the two series.

## RESULTS

*Normal Rat.* — 34 cases from the operations performed were accepted for final consideration. The discarded cases included animals, in which the implant sloughed after operation and cases in which the implant was buried in the entoderm mass, showing no contact with the reactive ectoderm. In all the useable cases a definite structure had been induced. As Diagram 1. shows, the induc-

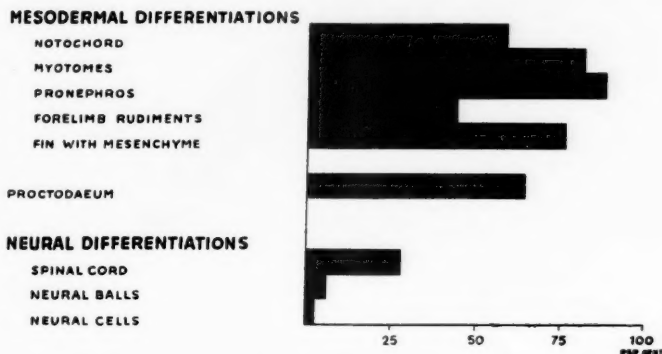


Diagram 1. — Diagram showing the percentages of the different structures induced by the bone-marrow of a normal rat. 34 cases.]

ing action of the bone-marrow of the rat was almost exclusively mesodermal. In 20 per cent of the cases neural structures were represented by a neural tube only, which without exception was very short and was always located at the tip of the tail. In addition, separate nerve cells or cell groups could be seen in 3 cases. True brain structures, in contrast, were never observed. A further frequent structure (22 cases) was the proctodaeum. It might be considered to be secondary formation induced by mesoderm (17).

*Leucemic Rat.* — On the same basis as the above, 33 cases were accepted for final consideration. Here the presence of the implant and its clear contact with the ectoderm was always established in the microscopic examination. Since the accepted larvae, in addition, were old enough (12—14 days) and the development was externally normal, it can be considered that the conditions for induction were the same as in the control material. In spite of this there were no cases of inductions. In 27 cases the inductor showed a broad surface of contact with the ectoderm and yet the latter showed no difference

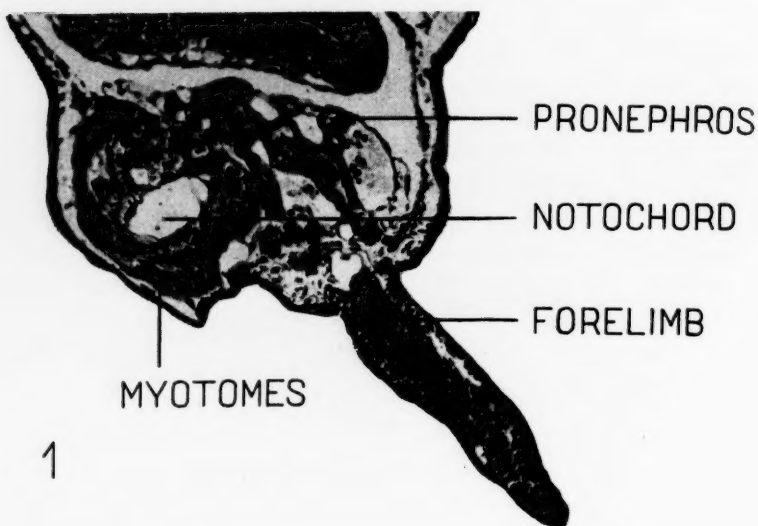


Fig. 1. — Photomicrograph showing the structures induced in a *Triturus* larva 14 days after implantation of a piece of bone-marrow of a normal rat.

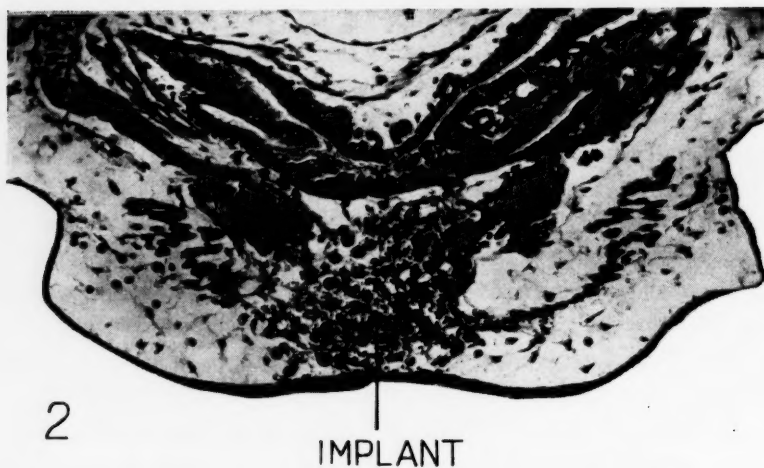


Fig. 2. — Photomicrograph showing an inactive inductor (leukemic bone-marrow) in the ventral region of a 14-days old *Triturus* larva. A large surface of contact between the inductor and the ventral epidermis is seen, but in the latter no reaction can be observed.



at the site of the inductor when compared with the normal ventral epidermis. In 6 cases the ectoderm showed a weak cell proliferation and thickening, but without any differentiations which would suggest a real induction. Thus one has to consider the leucemic bone-marrow used, in contrast to the normal tissue, as wholly inactive with regard to inductive action.

#### DISCUSSION

The number of operations performed gives, in our view, a sufficiently clear picture regarding the inducing action of the bone-marrow of the two individuals investigated. It is clear, however, that it is not possible to draw far-reaching conclusions regarding the inducing action of malignant tissues, since the investigation has been performed using one leucemic individual only.

The bone-marrow of the normal rat showed very similar inducing action to the earlier investigated bone-marrow of the guinea-pig (14, 15, 17, 18), i.e. purely mesodermal. The neural structures induced were somewhat more frequent than in the guinea-pig, but they always represented a weak neural reaction as compared with the mesodermal induction. The ratio neural agent/mesodermal agent is very low. As a result the neural structures are of caudal nature, as we have previously suggested (18).

The inactivity of the leucemic bone-marrow can hardly be due to experimental error, leading to the destruction of the inducing agent, nor to an erroneous technique. The very fact, that the similarly treated controls showed strong inducing actions argues against this. In addition it may be stated in this connection that no wholly inactive tissues have been found amongst the numerous kinds of fixed tissues whose inducing action has earlier been investigated (1, 13 etc.). Thus the leucemic bone-marrow shows a clear-cut difference from all normal tissues so far studied. The lack of the inducing power may, of course, be either primary or secondary. The latter possibility, however, cannot be analyzed in detail, since our knowledge of the chemistry of the mesodermal inductor and of processes taking place in malignant tissues is too scanty for such a discussion. It would be tempting to suppose, instead, that the lack of the inducing agent was primary and thus the reason for the increased growth and diminished differentiation

in the leucemic bone-marrow. Thus we would distinguish growth and differentiation as two separate events, regulated by different mechanisms. This way of thinking is by no means new. For example Needham (10) says: »It will be admitted that stimulus to differentiation is a very different thing from stimulus to uncontrolled growth.« According to this, the possibility would not be ruled out that when the inducing agents regulate the differentiation of tissues, they are at the same time suppressing excessive growth. The lack of inductors would lead to a disturbance of the balance between growth and differentiation, causing pathologically increased growth and decreased differentiation. It is, of course, questionable how far the induction processes occurring in the undifferentiated, embryonic tissue can be compared with the growth and differentiation phenomena in adult tissues. Some earlier experiments, however, suggest that such a comparison is warranted. Levander and his school (8, 9) were able to isolate a fraction from bone-marrow which is able to induce bone formation when injected into the muscle of an adult animal. When comparing these results with experiments performed with heterogeneous inductors, Levander (8, 9) and Schreiber (11) arrived at the conclusion that the same agents which in embryonic development regulate differentiation, also act in adult organisms, regulating their growth and differentiation, particularly regeneration. The earlier implantation experiments using alcohol-treated bone-marrow (14, 15, 16) lend support to this idea (see 16, pp. 242—243).

The investigations on the inducing effects of carcinogenic hydrocarbons, mentioned in the introduction (12, 19, 20), do not find explanation from the above-mentioned results and seem to be in conflict with the hypothesis presented here. Since these studies were performed in the late nineteen-thirties, opinions concerning the induction phenomenon and inducing agents have greatly changed. At the same time, one has the feeling that the connection of these two problems, malignant growth and embryonic induction, has been in a way based on a false assumption. Several investigations have shown (2, 3, 4, 6) that neural differentiation of the presumptive epidermis does not always presuppose the action of a specific inducing agent. On the contrary, it can be induced by numerous different chemical and even mechanical irritants. In this way, also, the experiments of Waddington (19) show that the

induction is caused by numerous chemical compounds, only some of which are carcinogens. Thus it seems that the inducing action of carcinogenic hydrocarbons could best be explained as unspecific toxic and cytolytic effects. Nor would they connect the effects of carcinogens and inducing agents as a common problem.

#### SUMMARY

The inducing action of the bone-marrow of a normal rat and a leucemic individual of the same strain has been studied using an implantation technique. The normal bone-marrow proved a strong and almost pure mesodermal inductor, whilst the bone-marrow of the leucemic rat was wholly negative as an inductor. The authors present as a hypothesis the possibility that the absence or inactivity of the inducing agent would lead to increased growth combined with weak differentiation.

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## THE EFFECT OF INACTIVATION OF THE SERUM ON THE GROWTH OF HELA CELLS<sup>1)</sup>

by

KARI PENTTINEN and ERKKI SAXÉN

(Received for publication April 9, 1956)

The effect of various active human sera on the growth of HeLa cells was studied in an earlier paper by the present writers, who observed differences between separata sera (2). The present study describes the effect of inactivation of human sera on the growth of HeLa cells. It was found that the inactivation of serum at 56° C increased the multiplication rate of the cells and also affected the size of the cell nuclei.

*Methods.* — Details of the method have been presented earlier (1). In the present investigation, however, mixture 199 was replaced by Hanks's solution in cultivating the cells before the experiment proper in Roux bottles. In the experiments the serum pools were used as 30 per cent dilution in Hanks's solution.

Inactivation was carried out in a water-bath. The temperature was carefully controlled, 56° C and 60° C being the temperatures used.

For enumeration of the cell nuclei four tubes were practically always used for each determination. Only those series where variation in the cell counts of inoculum was slight were accepted (2). Disintegrating and ghost nuclei were not counted.

The preparations for counting the mitoses and studying the nuclear changes were stained by the Feulgen nuclear method. To measure the nuclear sizes the preparations were projected on white paper, and the images of the nuclei were drawn. The final

<sup>1)</sup> Aided by a grant from the Sigrid Jusélius Foundation.

magnification on paper was 1000 x. The areas of the nuclei were measured by »Amsler Kompensations-Planimeter» type 612.

## RESULTS

One typical experiment on the effect of inactivation of the serum pool on nuclear counts is presented in Fig. 1.

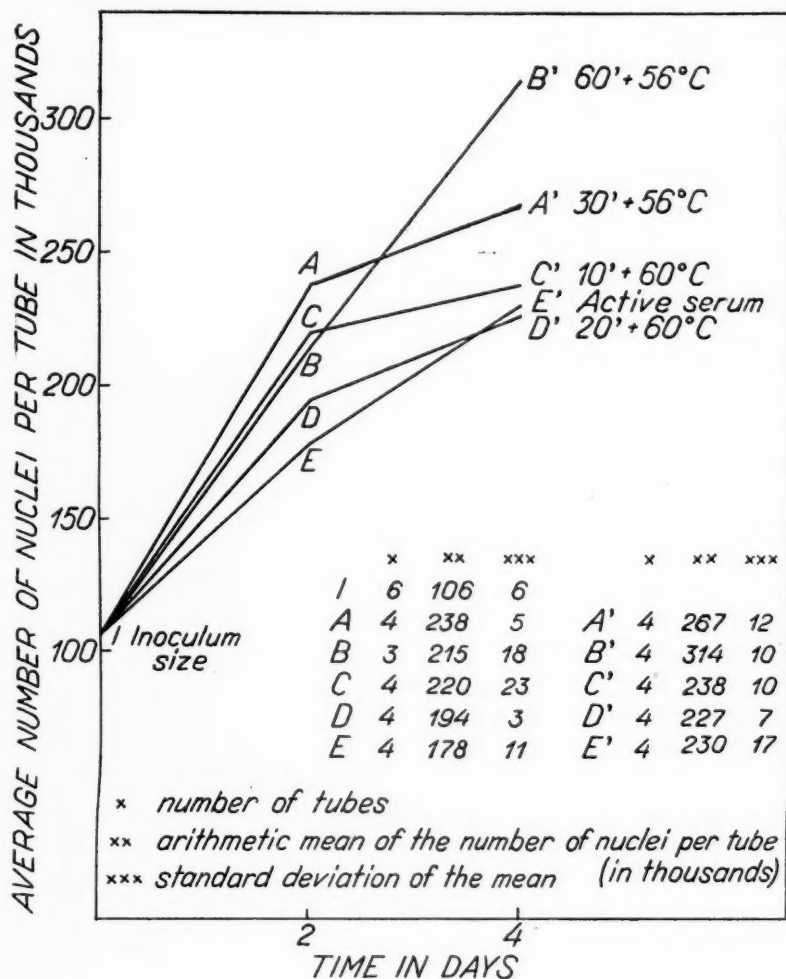


Fig. 1. — The effect of inactivation of the serum pool (three sera) on the growth rate of HeLa cells.

The results presented in Fig. 1 indicate that the inactivation of serum at 56° C tends to increase the growth rate when two- and four-day counts are taken into consideration. On increasing the inactivation temperature to 60° C, however, the cell counts decrease from the level obtained with inactivation at 56° C. The differences obtained between the two-day counts of active serum and serum inactivated at 56° C for 30 minutes, and between the latter and serum inactivated at 60° C for 20 minutes, were statistically significant (99 per cent significance level). Statistically significant was also the difference between the four-day counts of serum inactivated at 56° C for 60 minutes and active serum or sera inactivated at 60° C.

However, statistically significant (or insignificant) results of tissue culture experiments, like this, do not justify too far-reaching conclusions.

According to the results presented in the following chapter, the nuclear counts of the adjacent points C', D' and on the other side E' in Graph 1 have been reached in culture by different ways and thus the nuclear count alone does not give the right picture of the growth processes in different conditions.

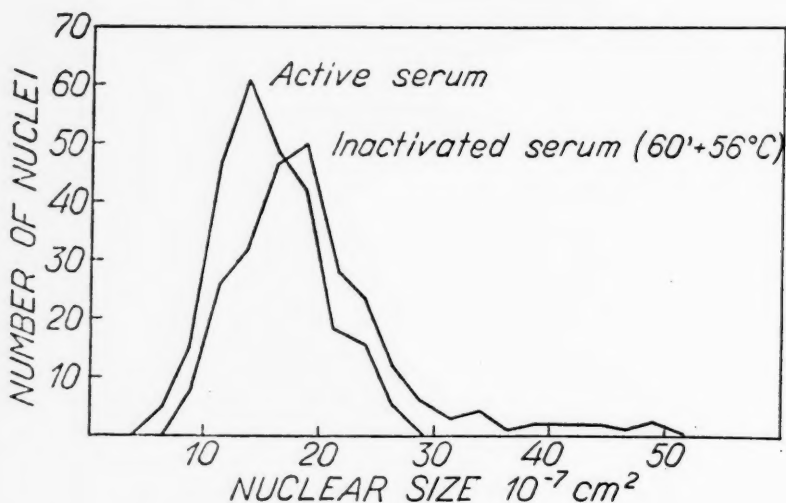


Fig. 2. — The distribution of nuclear sizes after four days growth in active serum and in serum heated at 56° C for 60 minutes.

*Nuclear Changes Due to Inactivation of Serum.* — In two-day counts the mitotic rate in cultures with inactivated serum was consistently higher than in cultures with active serum, but in four-day counts no differences were observed.

The percentage of degenerative changes in nuclei was about the same in cultures with active and inactivated sera at two-day counts, but after four days the percentage in the latter sera, especially in those inactivated at 60° C, was 2—3 times higher.

The nuclear size distribution in active and inactivated sera is presented in Graph 2.

Fig. 2 shows that in cultures grown in inactivated serum the average size of nuclei seems to be slightly greater and that the number of exceptionally large nuclei is also greater.

#### SUMMARY AND DISCUSSION

In our experiments with HeLa cells the criteria of growth followed, the number of nuclei, mitotic rate and nuclear size, tend to indicate that the cell growth is speeded up abnormally in suitably inactivated homologous sera. The question whether this is due to the destruction of the cell-growth controlling condition of active serum or to the production of a cell-growth stimulating condition by inactivation needs further study.

Lumsden et al, have in 1928 demonstrated that active heterologous sera have a cytotoxic effect in tissue culture which can be removed by inactivation. Homologous sera did not have such an effect. The observation that inactivation has an effect also when HeLa cancer cells are grown in human serum might have connection with the above mentioned phenomenon.

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## EFFECTS OF DIETARY CHOLESTEROL, DIETARY FAT, AND EXERCISE ON MOUSE PLASMA CHOLESTEROL

by

LEO PELTONEN and M. J. KARVONEN

(Received for publication April 11, 1956)

Mice have been used to a relatively small extent as experimental animals in studies of cholesterol metabolism. It appears, for instance, that the effects of cholesterol feeding on mouse plasma cholesterol have not been studied. On the other hand, the effects of diets rich in carbohydrate, fat and protein on the plasma cholesterol of hereditary obese-hyperglycemic and control mice have recently been investigated (5).

Claims have been made that physical activity has a tendency to suppress the serum cholesterol level in man (1), but the effects of exercise on cholesterol metabolism have not in general been satisfactorily investigated. In particular, the effects of exercise in the mouse are not known.

The purpose of the present study was to gain experience in the use of mice for studying cholesterol metabolism. The effects of dietary cholesterol, dietary fat, and exercise on the plasma cholesterol level were studied.

### MATERIAL AND METHODS

Three series of experiments were performed, using white mice.

*First Series.* — Female mice, from four to five months old, were used. They were divided in four groups: I, II, III, and IV. The

groups I and III were fed a standard diet the composition of which was as follows:

whole wheat flour .....	60	per cent
wheat germs .....	20	» »
casein .....	13	» »
margarine .....	4.8	» »
salt mixture .....	2	» »
cod liver oil .....	0.2	» »

The composition of the salt mixture was:

sodium chloride .....	49	per cent
calcium lactate .....	49	» »
ferric citrate .....	1.4	» »
manganese sulphate .....	0.49	» »
copper sulphate .....	0.10	» »
potassium iodide .....	0.01	» »

The groups II and IV were fed the standard diet to which 1.5 per cent cholesterol was added. All the groups were fed ad libitum.

The groups III and IV were exercised by making them swim in water at  $+36-37^{\circ}\text{C}$ . The experiment lasted 9 days. The mice swam on the first day for 30 min, and the exercise was increased progressively to 4 hours on the ninth day. From the third day on the daily exercise was divided in two portions, in order to reduce losses due to drawing. The total swimming time was 21 hrs 45 min.

A sample of blood was taken with a sharp glass pipette from the orbital venous plexus, on the ninth day from the beginning of the experiment, after anaesthetizing the mice with aether. The total plasma cholesterol was determined (6).

*Second Series.* — White male and female mice of the same breed and age were used as in the first series. Two grades of exercise and two concentrations of cholesterol in the diet were used, in addition to the control groups. In this way, nine groups of females and similarly nine groups of males were in the experiment. The plan of the experiment appears in Table 3.

*Third Series.* — A diet rich in fat but with a low cholesterol content was obtained by adding 20 per cent margarine to the standard diet. This was fed to male and female mice during ten days.

## RESULTS

*First Series.* — There were nine animals in each group. The mean plasma cholesterol levels of each group are shown in Table 1. The results of an analysis of variance are shown in Table 2. Feeding cholesterol caused a significant rise in the plasma cholesterol, and exercise suppressed the cholesterol level significantly. There was no significant interaction between cholesterol diet and exercise, i.e. cholesterol diet did not influence the effect of exercise or vice versa.

*Second Series.* — A full statistical analysis of this experiment was not made, because losses due to drawing reduced some of the groups to a very small number.

TABLE 1  
MEAN PLASMA CHOLESTEROL LEVEL OF FEMALE MICE, MG IN 100 ML OF PLASMA  
(FIRST SERIES)

	Normal Living	Exercise	Mean of All
Normal diet .....	65	59	62
Cholesterol diet .....	103	76	89
Mean of all .....	84	67	76

TABLE 2  
ANALYSIS OF VARIANCE (FIRST SERIES)

Source of Variation	Q	f	s <sup>2</sup>	v <sup>2</sup>	P
Differences of groups .....	10,031	3	3,344	6.26	0.01
Of it: effect of exercise .....	2,467	1	2,467	4.62	0.05
effect of diet .....	6,561	1	6,561	12.29	0.005
interaction of exercise and diet	1,003	1	1,003	1.88	> 0.1
Residual .....	17,090	32	534	.	.
Total	27,121	35	.	..	.

52 female mice lived to the end of the experiment. The mean plasma cholesterol and the standard error of the mean in each group are shown in Table 3. No regular relation between exercise and plasma cholesterol was observed, contrary to the results in the first series. Therefore, only the effect of cholesterol feeding was

subjected to an analysis of variance, without paying any attention to exercise. The results of the analysis of variance are shown in Table 4; they confirm the results of the first series.

TABLE 3

AVERAGES AND STANDARD ERRORS OF THE MEANS OF PLASMA CHOLESTEROL IN FEMALE MICE (SECOND SERIES)

Cholesterol Feeding		Normal Diet	1.5 Per Cent Cholesterol	3.0 Per Cent Cholesterol
Exercise				
Normal life	M .....	63	73	77
	st.e. ....	4.4	6.6	8.0
	n .....	7	8	5
Swimming 21 ½ hours	M .....	75	100	111
	st.e. ....	2.2	10.6	15.8
	n .....	5	7	4
Swimming 37 hours	M .....	66	88	96
	st.e. ....	2.2	7.7	8.1
	n .....	4	5	7

TABLE 4

PLASMA CHOLESTEROL IN FEMALE MICE (MG PER 100 ML OF PLASMA) AND ANALYSIS OF VARIANCE (SECOND SERIES)

	Control ( $x_0$ )	1.5 Per Cent Chol. Diet ( $x_1$ )	3.0 Per Cent Chol. Diet ( $x_2$ )
Mean .....	67.4	85.6	94.6

Source of Variance	Q	f	$s^2$	$v^2$	P
Between groups .....	6,171	2	3,086	6.92	0.01
Within groups .....	21,929	49	448	.	.
Total	28,100	51	.	.	.

Source of Variance	Q	f	$s^2$	$v^2$	P
$x_0 \dots (x_1, x_2)$ .....	5,320	1	5,320	11.88	0.01
Between $x_1$ and $x_2$ .....	851	1	851	1.90	> 0.05
Total between groups	6,171	2	3,086	6.92	0.01

The number of male mice remaining alive to the end of the experiment was 51. The variation of the plasma cholesterol appeared less regular and the scatter larger than in the females. No obvious relation between plasma cholesterol and exercise could be observed. Therefore, no analysis of variance was made. Disregarding exercise, the following mean values and standard errors of the means for each different diet were obtained:

	Control	Cholesterol: 1.5 Per Cent	3.0 Per Cent
Mean .....	93	91	88
Standard error of the mean	6.1	7.7	6.5
n .....	19	16	16

In the control group of male mice (no cholesterol feeding, no swimming) the mean plasma cholesterol was 87, st.d. 15 mg%. This is a higher value than the mean of the corresponding female group ( $p < 0.01$ ).

*Third Series.* — The results obtained with the diet rich in fat are shown in the following:

	Female		Male	
	Control	Fat	Control	Fat
Mean .....	63	77	87	81
Standard error of the mean ..	4.4	7.6	5.6	3.0
n .....	7	8	7	7

The differences did not attain statistical significance in either sex.

#### DISCUSSION

A high cholesterol diet caused a significant increase of plasma cholesterol in female mice in two series of experiments. This observation conforms to results obtained in several other species. However, the same was not seen in male mice. This may have been due to the primarily larger scatter of the plasma cholesterol values of the males, or it may reflect a true sex difference.

It has been previously reported (4) that in mice the whole blood cholesterol level is higher in the male than in the female. The present results with plasma cholesterol confirmed this finding.

A diet rich in fat caused no significant changes in the plasma cholesterol level. A similar observation has previously been made by Mayer and Jones (5); they also found that diets rich in carbohydrate or particularly in protein cause a suppression of the mouse plasma cholesterol.

In one series with exercise a suppression of the plasma cholesterol level was observed, but no effect in the other series. Environmental temperature and plasma cholesterol have been shown to be inversely related (7), but since the temperature of the water was in both series kept close to the body temperature, and since the mice also spent only a relatively short time in water, temperature can hardly have been responsible for the observed differences. Stress situations similarly as injections of ACTH are known to suppress the plasma cholesterol level (2, 3). No attempt was made to assess the role of adrenocortical stimulation in the present experiments. Physical exercise is often associated with symptoms of adrenocortical stimulation, and the possibility of an adrenocortical mechanism suppressing the plasma cholesterol level in connexion with exercise must be given serious consideration.

In further work with mice, particular attention ought to be paid to decreasing the scatter of the plasma cholesterol values. Whether this will be possible by taking repeated samples from the same animal and thus excluding some of the interindividual variation, remains to be seen.

#### SUMMARY

The effects of dietary cholesterol, dietary fat, and exercise on the plasma cholesterol level of white mice was studied.

An addition of 1.5 or 3.0 per cent of cholesterol to the standard diet caused a significant rise of the plasma cholesterol in female mice. A similar change could not be demonstrated in male mice.

An addition of margarine to the diet caused no changes in plasma cholesterol in either sex.

In one series exercise was followed by a significant fall of the plasma cholesterol in female mice. In another female series, how-

ever, no significant changes were observed in connexion with exercise. A series with males similarly did not indicate any change.

The plasma cholesterol level of control animals was significantly higher in the male than in the female.

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## UROPEPSIN EXCRETION IN NEUROCIRCULATORY ASTHENIA AND THYROTOXICOSIS

by

TEPPO VARTIO

(Received for publication May 5, 1956)

Uropepsin, a proteolytic enzyme of urine, is derived from the peptic cells of the gastric mucosa as a proenzyme, pepsinogen, which follows two pathways. The greater part is secreted into the gastric lumen where it is converted to active pepsin in the presence of hydrochloride acid, whereas the smaller portion diffuses from the peptic cells into the blood and is excreted in urine without being activated. Thus the determination of uropepsin provides evidence of the function of peptic cells (2).

It has also been said that the excretion of uropepsin is an evidence of the function of the adrenal glands (5). Thus it has been established that cortisone and ACTH raise the content of uropepsin considerably (3, 6).

As patients suffering from asthenia neurocirculatoria (AN) generally have gastric trouble and as it has been doubted wheetrs there are disturbances in the function of the adrenal glands, in thin disease, it seemed advisable to try to find out whether uropepshs showed any changes in this disease. As for the differential diagnosti of AN thyrotoxicosis is the main consideration, it seemed advisable to examine uropepsin excretion in this disease too.

### MATERIALS AND METHODS

The series studied consisted of 47 normal subjects (18 men and 29 women), 56 patients with neurocirculatory asthenia (16 men

and 40 women) and 20 patients with thyrotoxicosis, all of them women. The collection of normal patients and patients with AN and thyrotoxicosis was done simultaneously from 1. 9. 55 to 31. 3. 56. The personnel of the hospital were chosen as normal material together with some patients of the hospital who had completely recovered from acute infections and who were found to be free from other diseases.

As AN and thyrotoxicosis material were used the patients who were treated in the Provincial Hospital of Oulu at the time in question. The diagnosis of AN was established on the basis of a typical history and on the fact that in an objective examination nothing could be found to account for the troubles of the patient. By far the greatest part of the AN material had asthenic constitutions (41), only a small number were athletics (6) and those of no particular type were 9 in number. The diagnosis of thyrotoxicosis was established on the basis of typical objective symptoms, the most important of which are listed in Table III. All the patients had an enlarged thyroid, either diffusely or nodosely.

The uropepsin excretion was determined according to the method introduced by West, Ellis and Scott (7), with urine collected over a period of 24 hours.

#### RESULTS

The results appear in tables 1, 2 and 3. Normal values range between 13.5 and 58.3 units per hour. The mean value is 35.5 units per hour. In AN patients the values range between 14.2 and 66.1 units per hour. The mean value is 35.3 units per hour. In thyrotoxicosis the values range between 8.4 and 62.0 units per hour. The mean value is 34.8 units per hour.

#### COMMENTS

In this material, normal values of 13.5—58.3 units per hour with average 35.5 units per hour were obtained, which agrees with previous studies (3, 4, 6). The values obtained for AN patients and thyrotoxicosis do not differ from normal. According to what has been said above, it seems that the AN and thyrotoxicosis patients' pepsin excretion of gastric mucosa is normal. As in the gastric ulcer the uropepsin excretion has raised (1, 3), it seems, according

**TABLE 1**  
**UROPEPSIN EXCRETION IN NORMAL SUBJECTS**

[illegible]

**TABLE 2**  
**UROPEPSIN EXCRETION IN NEUROCIRCULATORY ASTHENIA**

No.	Age	Sex	Constitution	Uropepsin Excretion Rate Units/Hour
1	38	♂	athletic	38.1
2	38		asthenic	34.9
3	20		"	14.2
4	29		"	25.3
5	37		"	38.9
6	21		"	38.9
7	23		"	16.6
8	32		"	47.0
9	42		"	42.0
10	36		"	66.1
11	43		athletic	41.0
12	33		"	31.1
13	35		asthenic	25.8
14	30		no particular type	35.9
15	26		asthenic	60.1
16	33		"	61.7
17	22		"	35.0
18	29		"	31.5
19	24		"	36.2
20	43		athletic	31.7
21	35		no particular type	28.5
22	26		asthenic	39.1
23	41		"	35.8
24	24		"	44.8
25	31		"	24.4
26	27		"	42.0
27	37		"	53.9
28	36		"	44.8
29	30		"	28.5
30	27		"	31.0
31	24		"	25.3
32	32		"	34.9
33	23		"	14.5
34	31		"	26.4
35	52		"	39.1
36	30		"	31.3
37	37		no particular type	27.9
38	37		athletic	49.5
39	30		asthenic	42.0
40	30		"	42.6
41	31		no particular type	16.5
42	22		"	25.0
43	26		"	28.5
44	37		asthenic	29.0
45	34		"	17.8
46	24		"	24.5
47	26		no particular type	22.7
48	27		asthenic	31.4
49	31		no particular type	47.9
50	32		asthenic	37.0
51	39		no particular type	38.9
52	37		athletic	44.8
53	38		asthenic	19.7
54	36		"	33.3
55	26		"	61.6
56	30		"	39.1
				Mean 35.3

TABLE 3

## UROPEPSIN EXCRETION IN THYROTOXICOSIS

No.	Age	Sex	Basal Metabolic Rate %	Serum Cholesterol mg per 100 ml	Serum Protein- bound Iodine $\mu$ g per 100 ml	Uropepsin Excretion Rate Units/Hour
1	64	+	+ 59	225	16.8	25.8
2	28	+	+ 61	—	11.4	44.2
3	37	+	+ 70	—	16.8	24.0
4	48	+	+ 67	—	—	29.2
5	53	+	+ 73	218	—	8.4
6	44	+	+108	150	17.9	31.7
7	19	+	+ 80	216	—	37.0
8	55	+	+ 86	220	16.1	11.7
9	54	+	+ 93	175	28.0	34.9
10	40	+	+ 52	255	8.0	35.8
11	35	+	+ 54	—	—	27.9
12	25	+	+ 70	215	12.0	33.2
13	48	+	+ 75	210	9.8	26.9
14	37	+	+ 85	—	15.0	25.0
15	30	+	+ 71	220	—	33.3
16	52	+	+ 80	184	22.0	56.0
17	50	+	+ 34	220	12.4	31.0
18	41	+	+ 54	252	8.9	62.0
19	30	+	+ 65	—	14.5	57.3
20	40	+	+ 68	—	—	41.0
						Mean 34.8

to the above, as if there were no connection between this disease and the AN in this respect.

If the uropepsin excretion is regarded as an evidence of the function of the adrenal glands, it seems, according to this investigation, as if the uropepsin controlling function of the adrenal glands of the AN and thyrotoxicosis patients were not different from normal.

## SUMMARY

The uropepsin was determined from 47 normal subjects, 56 patients with neurocirculatory asthenia and 20 patients with thyrotoxicosis. The mean value obtained for the normal cases was 35.5 units per hour, 35.3 units per hour for the patients with neurocirculatory asthenia and 34.8 units per hour for patients with thyrotoxicosis. Thus the uropepsin excretion of the patients with neurocirculatory asthenia or thyrotoxicosis was not different from normal.

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## EFFECT OF CONTINUED IRON ADMINISTRATION ON THE ENDOCRINE GLANDS OF THE GUINEA-PIG

by

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(Received for publication May 16, 1956)

A search through the pertinent literature shows that the toxicity of various iron preparations has aroused increasing interest during the past years (3, review). In connection of our previous study dealing with the effect of cobaltous chloride on the thyroid of the guinea-pig (1) we found that the treatment with cobalt resulted in changes in some other endocrines, too, and these alterations became more pronounced when ferrous gluconate was injected together with cobaltous chloride (2). The study now reported was undertaken in an effort to investigate whether the administration of some iron preparations affects the endocrine glands of the guinea-pig.

### MATERIAL AND METHODS

Male guinea-pigs, weighing about 300 g, were used. The iron preparations were saccharated iron oxide (»Iviron», British Schering Ltd.), ferrous chloride ascorbate (»Ferroscorbin», Orion) and ferrous gluconate. Ten animals received intraperitoneal injections of »Iviron» in a daily dose of 80 mg Fe/kg, expressed in terms of iron, ten animals »Ferroscorbin» in a daily dose of 8 mg Fe/kg and ten animals ferrous gluconate in a daily dose of 20 mg Fe/kg. The treatment lasted 30 days. Ten littermates of the iron-treated animals served as untreated controls. The animals were killed by exsanguini-



nation, and the pituitary, thyroid, adrenal and thymus glands and the testes were removed, weighed with torsion balance, fixed in Bouin's fluid, embedded in paraffin and stained with Mallory's azan method.

# RESULTS

*Body Weight.* — The initial and final body weights of the animals were as follows:

	Controls (n = 10)	Iviron (n = 10)	Ferroskorbin (n = 10)	Ferrous gluconate (n = 10)
Initial Body Weight (g) .....	320 ± 4	314 ± 8	292 ± 8	314 ± 15
Final Body Weight (g) .....	520 ± 12	317 ± 26	360 ± 9	350 ± 20

The mean final body weight of the control animals differed highly significantly from those of the iron-treated groups.

*Pituitary.* — There were no statistically significant differences in the mean absolute and relative weight of the pituitary between the different groups. Neither revealed the histological examination any alterations in the pituitaries of the iron-treated animals.

*Thyroid.* — The mean absolute and relative weights of the thyroid and its histological picture in the three iron-treated groups were essentially similar to those of the controls.

*Thymus.* — The administration of iron caused a clear involution of the thymus in all the three groups. The mean absolute and relative weights of the thymus were as follows:

	Controls (n = 10)	Iviron (n = 10)	Ferroskorbin (n = 10)	Ferrous gluconate (n = 10)
Weights of the thymus (mg) ..	245 ± 26	89 ± 16	89 ± 11	48 ± 8
Weight of the thymus (mg/10 g)	48 ± 5	20 ± 3	25 ± 3	13 ± 1

The differences between the iron-treated groups and the control group were statistically significant.

*Adrenals.* — The mean absolute and relative weights of the adrenals were as follows:

	Controls (n = 10)	Iviron (n = 10)	Ferrosorbin (n = 10)	Ferrous gluconate (n = 10)
Weight of the adrenals (mg) ..	181 $\pm$ 16	190 $\pm$ 16	188 $\pm$ 10	160 $\pm$ 14
Weight of the ad- renals (mg/100 g)	35 $\pm$ 1	47 $\pm$ 4	53 $\pm$ 5	43 $\pm$ 2

The mean relative weights of the adrenals tended to be higher in the iron-treated groups, but a statistically significant difference was only between the Ferrosorbin-group and the control group. The histological structure of the adrenals of iron-treated animals was in general normal. In some cases, the cortical cells were hypertrophied and showed cytoplasmatic vacuoles.

*Testes.* — Especially ferrous gluconate resulted in a marked involution of the testes. Both the mean absolute weight of the testes ( $850 \pm 150$  mg) and their mean relative weight ( $240 \pm 29$  mg/100 g) differed significantly from those of the controls ( $2086 \pm 81$  mg and  $402 \pm 20$  mg/100 g, respectively). A detailed microscopical examination revealed that the seminiferous tubules were narrow, the germinal epithelium was atrophied, the number of spermatogonia was decreased and the maturation division was interrupted at the pachytene stage of prophase. Metaphases and anaphases could not be seen, neither mature spermia. In the two other iron-treated groups changes were found only in some cases.

#### DISCUSSION

According to our results, the treatment of guinea-pigs with saccharated iron oxide, ferrous chloride ascorbate and ferrous gluconate in the doses used resulted in within one month a marked atrophy of the thymus, a slight hypertrophy of the adrenals, and, regarding ferrous gluconate, an involution of the testes with strongly deranged spermiogenesis. No changes were found in the pituitary and in the thyroid. The weight gain of the iron-treated animals was much slower than that of controls.

Nissim (4), when studying whether the administration of ferric hydroxide ferrous ascorbate affects the growth, adrenal and pituitary glands of guinea-pig, found a depressed growth and adrenal damage, findings, which are in agreement with the present

observations. He observed also degenerative changes in the pituitary, an observation, which could not be found in the conditions of the present experiment.

Little is known on the actual mechanism of the iron poisoning. A profound shock has been observed as the cause of death in acute iron poisonings. Smith (5) believes that the excess of iron destroys the mucosal barrier and excessive amounts will enter to mucosal cells. This may escape into the circulation. Further, the excessive serum iron may be converted into ferritin in the liver, spleen and bone marrow, and find its way into the circulation. Ferritin is known to be a factor in producing a shock-like syndrome. Thus, the changes observed in the adrenals and thymus can be considered as a counter-shock phenomenon.

#### SUMMARY

Saccharated iron oxide, given in intraperitoneal injections in a daily dose of 80 mg Fe/kg, ferrous chloride ascorbate, in a daily dose of 8 mg Fe/kg and ferrous gluconate in a daily dose of 20 mg Fe/kg resulted in within a month a marked involution of the thymus and a slight hypertrophy of the adrenals of guinea-pig. Ferrous gluconate caused a pronounced involution of the testes and an interrupted spermiogenesis. There were no changes in the histological picture of the pituitary and thyroid glands.

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FROM THE UNIVERSITY CLINIC OF HELSINKI TUBERCULOSIS SANATORIUM  
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## EFFECT OF CORTISONE ON TUBERCULIN AND HEMAGGLUTINATION REACTIONS AND SERUM PROTEIN

OBSERVATIONS MADE IN THE COURSE OF ANTITUBERCULOUS  
MEDICATION

by

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(Received for publication May 10, 1956)

The favourable effect of cortisone and ACTH treatment on both the general and topical symptoms of infections has long been known. In patients with pulmonary tuberculosis, tuberculine sensitivity has been found to decrease during cortisone treatment and increase again after treatment (5, 6). The hemagglutination reaction titre is reported, in some cases, to rise after cortisone and ACTH treatment (5, 6). Some investigators (2, 9) report that cortisone and ACTH therapy affects the protein fractions of the blood of patients with pulmonary tuberculosis, primarily by increasing the serum albumin and albumin globulin ratio. In some cases the gamma globulin shows a clear rise after the ACTH treatment in concluded. As the study of these points has been limited to occasional observations only, the purpose of the present work is to throw light on the effect of cortisone treatment on the above reactions in individuals suffering from pulmonary tuberculosis. It is hoped in this way to provide a more reliable picture of the effect of cortisone on the mode of reaction and resistance of the organism in tuberculous patients.

The series comprised 12 patients whose ages ranged from 31—51 years. Two of them were women. All had far advanced pulmonary tuberculosis.

The *investigations* were carried out as follows:

(I) During antituberculous treatment just before starting the cortisone treatment,

(II) 10 days after the initiation of cortisone treatment,

(III) 10 days after the completion of cortisone treatment.

1. The *Mantoux tuberculin tests* were effected as usual with 0.01 and 0.001 mg of OT and read after 72 hours.

2. The *hemagglutination reactions* were made by the State Serum Institute, as modified by Dubos-Middlebrook (3). The lowest positive titre was 1: 16.

3. *Total serum proteins* were determined photometrically by the biuret method.

4. The *electrophoretic determinations* were carried out with Antweiler's micro-electrophoresis apparatus. Dole's veronal, veronal-sodium buffer was used: ionic strength 0.12, pH 8.6. Dialyzing time was 2—4 hours. The current was 1.8 mA, running time 18 min. Double determinations were made of each serum. In Antweiler's apparatus the  $\alpha_1$ -globulins join the albumin fraction. The normal values were taken to be: Albumin +  $\alpha_1$ -globulins 68 per cent,  $\alpha_2$ -globulins 5 per cent, beta-globulins 12 per cent, and gamma-globulins 15 per cent (1).

The following *antituberculous treatment* was given to the patients during the investigation: Antituberculous drugs (streptomycin, isoniazid and PAS) were administered for two weeks or more before the initiation of cortisone treatment (tabl. Cortisate/Leo 25 mg) and continued for a minimum of two weeks after the cortisone treatment. The total cortisone dose was 1 g, administered over a period of 20 days. The initial dose was 125 mg/day, decreased gradually to 12.5 mg/day.

## RESULTS

The *Mantoux reactions* varied from nil to 204 sq mm. In 7 cases the reaction was smaller during than before cortisone therapy, in 2 cases the same and in 3 higher. The average reaction before cortisone treatment was 86.6 sq mm, during the treatment 65 sqmm, and after treatment 104 sq mm. These changes were not statistically significant.

The results of the *hemagglutination reactions* are given in Table 1.

TABLE 1  
HEMAGGLUTINATION REACTIONS

Case No.	Hemagglutination Reaction Titre		
	Before Cortisone Treatment	During Cortisone Treatment	After Cortisone Treatment
1	1/64	1/64	1/64
2	1/64	1/32	1/32
3	1/32	1/16	1/8
4	1/16	1/16	1/16
5	1/64	1/16	1/64
6	1/64	1/64	1/4
7	1/32	1/8	1/8
8	1/16	1/8	1/16
9	1/64	1/2	1/32
10	1/16	1/8	1/64
11	1/32	1/16	1/16
12	1/16	1/8	1/16
Average	1/28.5	1/9.2	1/14

The reactions ranged from a negative  $\frac{1}{2}$  to a strongly positive 1/64 (no higher titres were studied). In 9 cases the titre was lower during than before the treatment, and in 3 cases it was the same. The titre seemed to rise again after cortisone treatment: in 5 cases the titre was higher after the end of cortisone treatment than during it, in 5 the same and in 2 lower. The mean values before cortisone were 1/28.5, during the treatment 1/9.2, and after it 1/14. These changes are not statistically significant ( $t = 1.93$ ,  $P > 0.05$ ).

TABLE 2  
MEAN PROTEIN VALUES

	Before	During	After	Mean Value,		t	t	P	P
	Cortisone Treatment			Difference					
	A	B	C	A-B	B-C				
	g/100 ml	g/100 ml	g/100 ml	g/100 ml	g/100 ml	A-B	B-C	A-B	B-C
Total protein ..	6.76 ± 0.75	7.56 ± 0.64	7.13 ± 0.50	0.8	0.43	2.61	1.85	<0.05	>0.05
Albumin + α <sub>1</sub> -globulin ..	3.29 ± 0.77	4.01 ± 0.63	3.67 ± 0.74	0.72	0.34	2.50	1.21	<0.05	>0.05
α <sub>2</sub> -globulin ....	0.92 ± 0.24	0.88 ± 0.21	0.88 ± 0.22	0.04	—	0.42	—	>0.05	—
β-globulin ....	0.70 ± 0.18	0.92 ± 0.29	0.82 ± 0.20	0.22	0.10	2.23	0.98	<0.05	>0.05
γ-globulin ....	1.86 ± 0.32	1.76 ± 0.30	1.77 ± 0.31	0.10	0.01	0.78	—	>0.05	—
alb/glob. ....	0.98 ± 0.34	1.16 ± 0.30	1.09 ± 0.34	0.10	0.07	1.34	0.52	>0.05	>0.05

The total serum protein values varied from 5.5–8.6 g%. During the cortisone treatment the total proteins rose by an average of 0.8 g%, a statistically significant difference at the 5 per cent level. After discontinuation of cortisone the total protein values dropped again, although the drop was not statistically significant.

Of the individual serum protein fractions, the increase occurred mainly in the *albumin- and  $\alpha_1$ -globulin fractions*, which increased by an average of 0.72 g %, a result statistically significant at the 5 per cent level. After the discontinuation of cortisone there was a drop in the fraction, though not statistically significant. The  *$\beta$ -globulin amount* increased during the treatment by 0.22 g %. a statistically significant rise at the 5 per cent level. No statistically significant changes were noted in the  *$\alpha_2$ - and  $\gamma$ -globulins*, although a very slight drop occurred in both fractions during cortisone treatment. The *albumin-globulin ratio* seemed to rise during cortisone treatment although the change was not statistically significant.

#### DISCUSSION

A reduction in the tuberculin sensitivity during cortisone treatment was noted in over half the cases of the material. The result, thus, complies fairly well with the findings made in human and animal tuberculosis (5, 6, 7).

The hemagglutination titre seemed generally to drop during cortisone treatment although the average reduction in titre was not statistically significant. LeMaistre et al. showed that, although some cases might show a rise in the titre after treatment, neither ACTH nor cortisone therapy had any clear effect on the titre. This, in our opinion, may be due to the aggravation of the tuberculosis.

The total serum protein, often lowered in tuberculosis (8, 10), seemed from our investigation to rise during cortisone treatment, a finding compatible with the experience gained in connection with other diseases and reported in the literature (4).

Electrophoretically, an increase was noted during cortisone treatment in the albumin and  $\alpha_1$ -globulin and in the  $\beta$ -globulin fractions. On the other hand, no appreciable changes were recorded for the  $\alpha_2$ - and  $\gamma$ -globulin fractions. The albumin-globulin ratio showed a normalization during cortisone treatment although the rise was not statistically significant.



It seems possible that especially the changes in the electrophoresis and tuberculine sensitivity would have been clearer had the investigations been made within a few days of the treatment, while the cortisone effect was at its maximum subjectively. The evaluation of the cortisone effect is also complicated by the fact that the condition of some of the patients improved during the treatment; in these cases, naturally, normalization of the serum fractions was attributable to some extent to their improved condition, and for this reason the fractions never returned to their pre-treatment level (11).

#### SUMMARY

The authors investigated the effect of cortisone administered orally during antituberculous medication on the tuberculin and hemagglutination reactions of 12 patients with far advanced pulmonary tuberculosis, and on their electrophoretically determined serum fractions. During cortisone treatment, the tuberculin reactions showed a reduction in sensitivity and the hemagglutination reactions a drop in titre; however, the changes were not statistically significant. The total serum protein, albumin- (and  $\alpha_1$ -globulin) and  $\beta$ -globulin fractions seemed to increase during cortisone treatment, statistically significantly at the 5 per cent level. In no other fractions were definite changes observed.

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## STUDIES ON THE HOST-PARASITE RELATIONSHIP

### I

#### INFLUENCE OF DIPHTHERIA-PERTUSSIS-TETANUS VACCINE ON GUINEA- PIG TUBERCULOSIS

by

OLE WASZ-HÖCKERT and ALF BACKMAN

(Received for publication May 16, 1956)

The present investigation<sup>1</sup> was carried out as a continuation of our previous research into the effect of various vaccines on experimental guinea-pig tuberculosis (6, 7). The basis of this and the earlier investigations consists of clinical observations by Wasz-Höckert (1953, 1954) (4, 5) of the flaring-up of meningitis or the appearance of persistent infections in children after vaccination against smallpox, pertussis-diphtheria or tuberculosis, and by Backman & Wasz-Höckert (1954) (1) on the connection between tuberculosis of the bones and joints and the above vaccinations.

The investigations I and II follow the lines stated below and expressed schematically in Fig. 1.

1. Study of the stress effect with polyvalent vaccine composed of three antigens (Diphtheria-Pertussis-Tetanus) compared with the effect of a univalent vaccine (Pertussis).

2. Difference in effect between repeated stresses and a single stress.

3. Difference in effect between stress before challenge, in connection with the challenge, and some time after the challenge.

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

4. By using bacillus doses of varying sizes, study of how a difference in the severity of the challenge affects the course of the infection.

Part I consists of two series of experiments schematically expressed in Fig. 1.

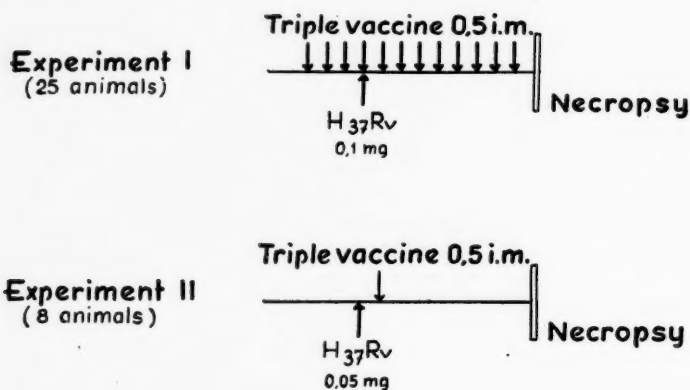


Fig. 1

*Experiment I.* — The guinea-pigs were exposed to stress with Diphtheria-Pertussis-Tetanus vaccine (triple vaccine) two weeks prior to TB-inoculation and the twice a week during the course of the experiment.

*Experiment II.* — The guinea-pigs were exposed once only to stress with triple vaccine. The dose, the same as the above, was given 1 week after TB-inoculation, which in this case was done with a smaller dose tubercle bacilli than in experiment I.

#### EXPERIMENT I

*Material and Method.* — Experimental animals: 27 guinea-pigs, average weight 420 g, were employed. Before the experiment, the animals were tested with a Mantoux test (1.0 mg of Old Tuberculin) and were found to have a negative tuberculin reaction.

**TB inoculation:** The animals were inoculated in the right inguinal fold with 0.1 cc of a *M. tuberculosis* suspension containing 0.1 mg of H<sub>37</sub>Rv bacilli per 0.1 cc. (The amount of bacilli was determined from Dubos medium according to the centrifuging method.)

The animals were divided into two main groups:

Group A.— Control group, no treatment after  $H_{37}Rv$  challenge (10 animals).

Group B — The animals were treated with 0.5 cc of triple vaccine (diphtheria-pertussis-tetanus vaccine)<sup>1</sup> intramuscularly twice a week. This treatment was started two weeks prior to  $H_{37}Rv$  inoculation and continued for the course of the experiment (15 animals).

In addition, two healthy animals were treated with diphtheria-pertussis-tetanus vaccine in the same way as the animals of Group B.

*Results.* — The results of the experiment were as follows:

1. As can be seen from Table 1, the reversal of the tuberculin allergy (measured by Mantoux 1.0 mg test) was approximately similar in both groups.

TABLE 1

MANTOUX 1.0 MG PERFORMED 14, 21 AND 28 DAYS AFTER THE  $H_{37}Rv$  CHALLENGE. THE MANTOUX REACTIONS, READ AFTER 3 DAYS, ARE INDICATED AS —, ±, OR +

Group	Mantoux Reaction, Days after TB-Inoculation								
	14			21			28		
	—	±	+	—	±	+	—	±	+
A	10	—	—	8	2	—	—	—	10
B	15	—	—	10	4	1	—	3	12

2. The weight curve of Fig. 2 shows the clear difference between the two groups of animals during the experiment. In the group exposed to stress with triple vaccine the weight gain was considerably smaller than in the control group.

3. Necropsy findings. All the experimental animals were sacrificed and necropsied 31 days after the  $H_{37}Rv$  inoculation. The necropsy was performed «blindly» in that the examiner did not know to which group the animal belonged. The inoculation site, the regional glands, lungs, liver and spleen were examined macroscopically, and the results were plotted diagrammatically (Fig. 3).

<sup>1</sup> Diphtheri-Pertussis-Tetanus Impfstoff Behringwerke (DPT)  
30,000 million pertussis bacilli per 1 ml.  
Minimum 50 Di.S.U. per ml.  
Minimum 50 Tet.S.U. per ml.

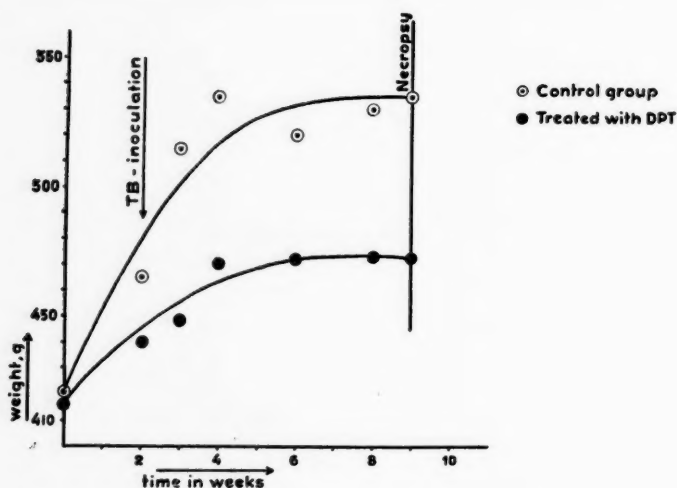


Fig. 2

The figure shows clearly that both the number and size of the tuberculous lesions in the organs examined were larger in the group subjected to stress with triple vaccine.

The results were also computed according to Feldman's principle (2, 3), with the following findings:

TABLE 2

	Spleen	Lungs	Liver	Inoculation	Total
Group A (Control)	10	0	4	10	24
Group B (triple vaccine)	20	0	10	10	40

The tuberculous lesions in the organs listed in Table 2 were evaluated as follow:

	Large Changes	Moderate Changes	Slight Changes
Spleen .....	35	20	10
Lungs .....	30	20	10
Liver .....	25	20	10
Inoculation .....	10	10	10

On this basis, the results have been computed and averages given for both groups.

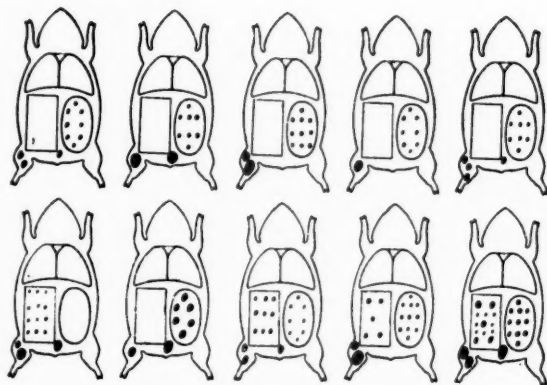
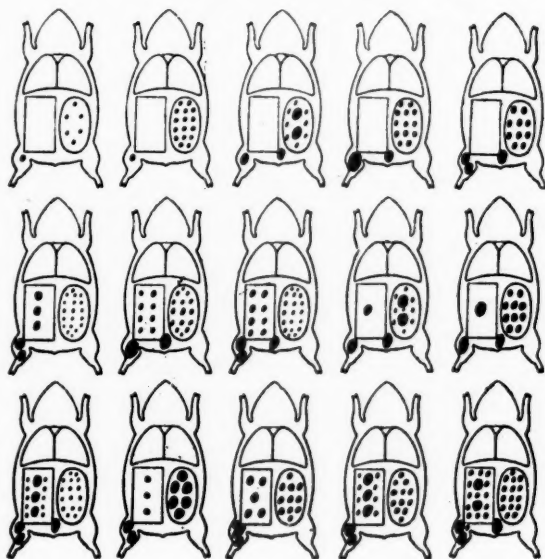
**CONTROLS****TREATED WITH TRIPLE VACCINE**

Fig. 3



As can be seen from Table 2, a distinct majority of the lesions found were stated in Group B (treated with triple vaccine).

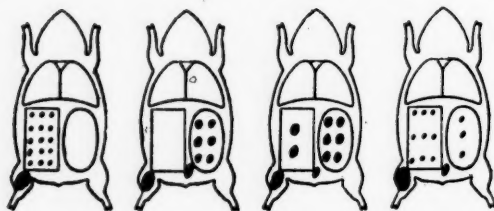
The two experimental animals treated solely with triple vaccine revealed no special changes on necropsy.

#### EXPERIMENT II

*Material and Method.* — Eight Mantoux-negative (1.0 mg of Old Tuberculin) guinea-pigs, average weight 405 g, were challenged with 0.05 mg of  $H_{37}Rv$  bacilli (the amount was determined as for Experiment I). The animals were divided into two groups of 4 each. Group A was kept as a control. The animals of Group B were given 0.5 cc of the triple vaccine (Diphtheria-Pertussis-Tetanus vaccine) intramuscularly 7 days after the tubercle bacilli challenge. 30 days after inoculation the animals reacted positively to tuberculin (1.0 mg of Old Tuberculin), were sacrificed and necropsied.

*Results.* — The results of the necropsies, computed as for Experiment I, are given in Fig. 4.

#### CONTROLS



#### TREATED WITH TRIPLE VACCINE

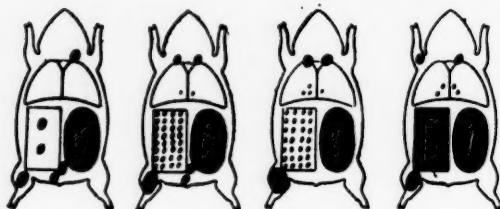


Fig. 4

As can be seen from the above figure, both in number and size the tuberculous lesions were larger in the group exposed to stress with the triple vaccine (Group B).

The result was also computed according to Feldman's principle (see Experiment I), giving the following results:

TABLE 3

	Spleen	Lungs	Liver	Inoculation	Total
Group A (Control)	10	0	7	10	27
Group B (triple vaccine)	35	0	19	10	71

Table 3 shows that a definite numerical majority of the tuberculous lesions were stated in Group B (animals treated with triple vaccine).

No difference was observable between the two groups as regards the increase in bodyweight of the animals or reversion of tuberculin allergy.

## DISCUSSION

In *Experiment I* the results reveal a distinct aggravation of the infection among the animals treated with the vaccine, compared with the control material. This difference is not so marked as it was in our earlier investigations or in the other experiments of the present work. One of the causes, we believe, is that the animals, having received antigen before inoculation, were stimulated to increase antibody formation. Hence, at the moment of inoculation, they were so well placed to fight the induced infection that a later stress could not affect the course of the infection as adversely as is usually the cases.

In *Experiment II*, again, the difference between the control group and the animals exposed to stress with a post-inoculation injection of DPT vaccine is distinct. This in spite of the fact that the inoculation dose was smaller than in Experiment I and although the post-inoculation stress was slighter. The result is attributable to the fact that the antibody formation of the animals was in no way mobilized before the challenge; for this reason even a slighter infection could cause changes of the same and even greater degree than the larger bacillus dose of Experiment I.

The results to the questions presented in the beginning of the paper, will be further discussed in part two (8) of this study, since experiments III and IV are reported there.

#### SUMMARY

The writers have investigated the effect of diphtheria-pertussis-tetanus vaccine on resistance to a tuberculous infection in guinea-pigs. A marked difference was shown between the groups exposed to stress and those not subjected to stress with vaccines.

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## STUDIES ON THE HOST-PARASITE RELATIONSHIP

### II

#### INFLUENCE OF PERTUSSIS VACCINE ON GUINEA-PIG TUBERCULOSIS

by

OLE WASZ-HÖCKERT and ALF BACKMAN

(Received for publication May 16, 1956)

To complete our earlier investigations (10, 12) a study divided into four experiments has been performed, of which two are reported in this issue, concerning the influence of diphteria-pertussis-tetanus vaccine on the resistance to an experimental *M. tuberculosis* infection in guinea-pig (13).

The principles for this investigation<sup>1</sup> can be explained most clearly by the following diagram:

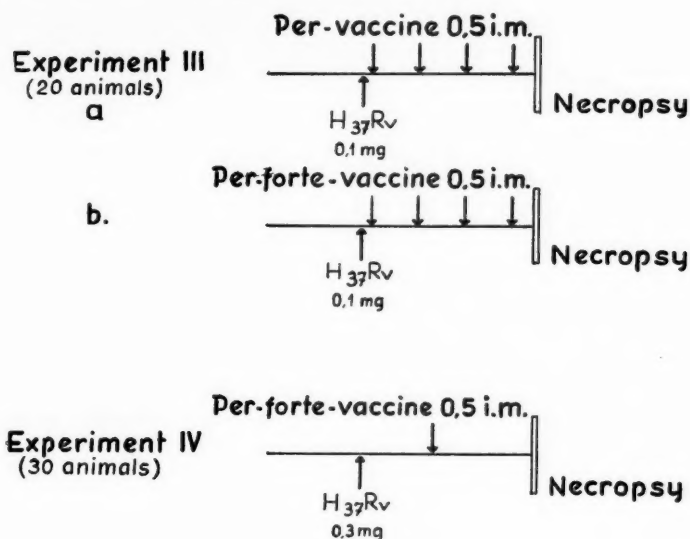


Fig. 1.

<sup>1</sup> Aided by a grant from Sigrid Jusélius Foundation.

By way of explanation to the figure, the following is pointed out:

*Experiment III:* The guinea-pigs were exposed to stress with Pertussis or Pertussis forte vaccine, respectively, three days after the challenge with bacilli (the same dose as in Experiment I, and once a week throughout the course of the experiment).

*Experiment IV:* The guinea-pigs were inoculated with a larger dose of TB bacilli than in Experiments I—III and were exposed to stress with one injection of Pertussis forte vaccine 13 days after the challenge.

### EXPERIMENT III

*Material and Method.* — The series comprised 20 guinea-pigs with a negative Mantoux reaction (1.0 mg of Old Tuberculin), average weight 250 g. The animals were inoculated subcutaneously in the right inguinal fold with 0.1 mg of H<sub>37</sub>Rv bacilli (The amount of bacilli was determined from Dubos medium according to the centrifuging method).

The animals were divided into three groups:

Group A — Control (5 animals).

Group B — The animals were exposed to a stress with one intramuscular injection of 0.5 cc of Per-vaccine<sup>1</sup> on the third day after the TB challenge and thereafter once a week to a total of four injections (5 animals).

Group C — The animals were exposed to a stress with a intramuscular injection of 0.5 cc of Pertussis forte vaccine<sup>2</sup> on the third day after the TB challenge and thereafter once a week to a total of 4 injections (10 animals).

Five weeks after the challenge with H<sub>37</sub>Rv the animals reacted positively to Mantoux (1.0 mg of Old Tuberculin), were sacrificed and necropsied.

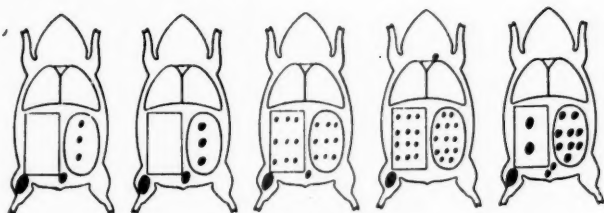
*Results.* — The results of the necropsies, presented as for Experiment I (13) are shown in Fig. 2.

As can be seen from the figure, both the number and size of the tuberculous lesions were greatest in Group C (treatment with

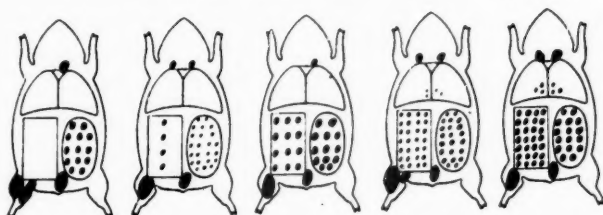
<sup>1</sup> Per vaccine «Orion»: 1 ml contains 2,000 million bacilli. 0.01 per cent of Merthiolate Eli Lilly was used as preserving agent.

<sup>2</sup> Pertussis forte «Orion»: 1 ml contains 15,000 million bacteria. 0.01 per cent of Merthiolate Eli Lilly was used as preserving agent.

# CONTROLS



# TREATED WITH PERTUSSIS VACCINE



# TREATED WITH PERTUSSIS FORTE VACCINE

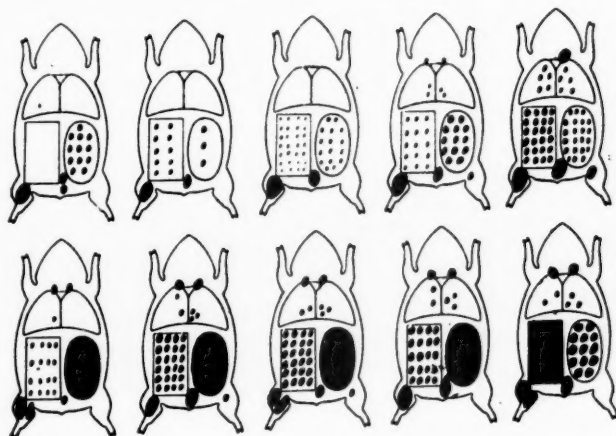


Fig. 2.

Pertussis forte vaccine), somewhat smaller in Group B (treatment with Pertussis vaccine), and smallest in the untreated control material.

The results were also computed according to Feldman's principle (2, 4).

TABLE 1

	Spleen	Lungs	Liver	Inoculation	Total
Group A (control) .....	14	0	6	10	30
Group B (Pervaccine) .....	26	4	16	10	56
Group C (Perforte vaccine) .....	26	9	19	10	64

As can be seen from Table 1, a definite numerical majority of tuberculous lesions occurred in Group C (animals exposed to stress with Per Forte vaccine). The animals of Group B (stress with Pervaccine) also showed considerably more marked changes than the animals of the control group (Group A).

No difference was observable between the different groups in the increase in bodyweight of the animals or the reversal of tuberculin allergy.

## EXPERIMENT IV

*Material and Method.* — The series comprised 30 guinea-pigs with a negative Mantoux reaction (1.0 mg of Old Tuberculin), average weight 355 g. They were inoculated subcutaneously above the sternum with 0.3 mg of H<sub>37</sub>Rv bacilli (the amount of bacilli was determined as for Experiment I). The animals were divided into two groups.

Group A. — Control (15 animals).

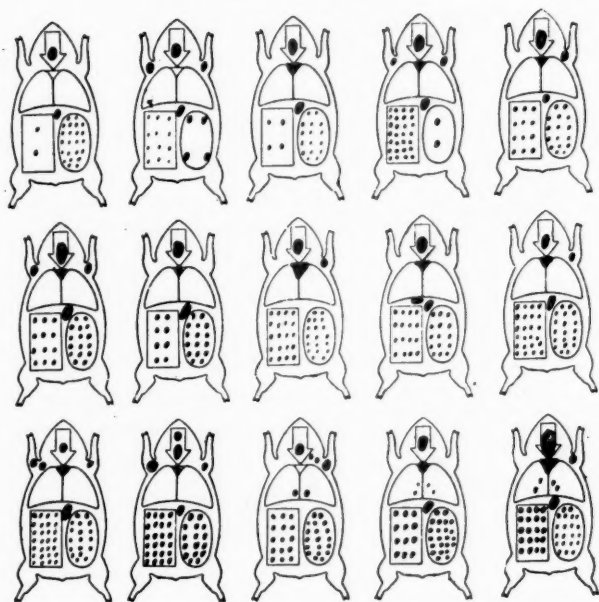
Group B. — The animals were exposed to a stress with one intramuscular injection of 0.5 cc of Pertussis forte vaccine 13 days after the TB challenge (15 animals).

Four weeks after the H<sub>37</sub>Rv challenge the animals reacted positively to Mantoux (1.0 mg of Old Tuberculin), were sacrificed necropsied and blood-samples were taken for serological investigations.

*Results.* — The results of the necropsies are:



## CONTROLS



## TREATED WITH PERTUSSIS FORTE VACCINE

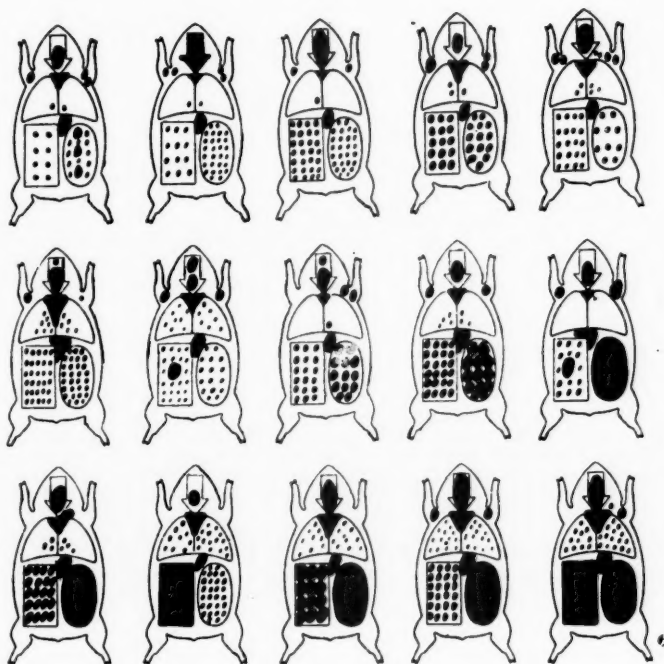


Fig. 3.

Fig. 3 shows that the tuberculous lesions are markedly more severe among the animals of Group B (exposed to stress with Pertussis Forte vaccine) than among those of Group A (control).

The results have also been computed according to Feldman's principle (2, 4).

TABLE 2

	Splee	Lungs	Liver	Inoculation	Total
Group A (control) .....	18	2	16	10	46
Group B (Perforte vaccine) .....	26	13	20	10	69

Table 2 reveals that, computed according to Feldman's principle, a definite numerical majority of the tuberculous lesions occurred among the animals of Group B (stress with Per forte vaccine) compared with Group A (control).

A comparison of the animals of the two groups reveals no difference in the increase of bodyweight or reversal of tuberculin allergy.

In this experiment a blood sample for serological testing was taken at autopsy from each guinea-pig by cardiac puncture. The *Middlebrook-Dubos hemagglutination test* and the *Middlebrook hemolysis test* were made. No statistical difference was seen in either test on comparison of the control group (13 animals) and the Pertussisforte treated group (13 animals), although 53% and 61% respectively of the animals show an increase over the normal (1/8) in the tests.

#### DISCUSSION

In *Experiment III* the difference between the three groups of animals was distinct. The vaccine containing most antigen (Pertussis forte) caused more marked changes than the vaccine of lower antigen content (Pertussis). A strong univalent vaccine, injected after the challenge, so engaged the host's antibodies that the induced infection was given freer play.

In *Experiment IV* in which a large inoculation dose was employed and the stress effect was obtained with a strong, univalent vaccine (Pertussis forte) two weeks after the inoculation, the

difference between the animals exposed to stress and the control animals was very marked. Here a strong, univalent vaccine, injected at an advanced stage of the infection, so engaged the antibodies that the lesions caused by the infection were greatly aggravated. The bacillus dose employed, large compared with the other experiments, probably equals the dose of TB bacilli guinea-pigs are given in routine examinations of sputum from patients with clinically severe pulmonary tuberculosis.

In both part I (13) and the present paper, the points we wish to bring out are the following:

In our previous investigations we have been able to show that the vaccines we used (BCG, Pertussis-Diphtheri-Variola and Pertussis) aggravated guinea-pig tuberculosis. That heterologic vaccines in large doses have a distinct stress effect on infection of chronic type was thus demonstrated. No similar investigations have been reported in the literature before, but Gerniez-Rieux & al. (3) showed (1951) that typhoid-paratyphoid vaccine (TAB) affected the survival period of tuberculous guinea-pigs. Parfentjev (1953) (7) showed that mice injected with Pertussis vaccine were killed by a smaller dose of living bacteria (*Proteus*, *Brucella*, *Pasteurella*) than those not treated with vaccine.

Volkert, Pierce, Horsfall & Dubos (1947) (14) showed that influenza virus A aggravated the pulmonary changes in experimental mouse tuberculosis. In our earlier investigations (10, 12) we found that virus-stress (Variola vaccine) had a strongly aggravating effect on experimental guinea-pig tuberculosis. The well-known clinical observation that tuberculin allergy disappears and resistance to tuberculous infection is reduced in connection with measles is probably related with this mechanism.

As was pointed out in the discussion at the Pediatric Congress in Oslo in 1954 (11), the doses of vaccine we used in our experiments bear no relation to those used in pediatric practice. We have injected 0.5 ml into guinea-pigs weighing up to 400 g, i.e. the dose for a child of 5–20 kg.

We have no intention of discrediting the vaccinations. What we want to show is how well the vaccines act as stress factors for studying the course of experimental infection, particularly the host-parasite relationship. We must, however, emphasize the importance of the fact that children to be vaccinated must be

perfectly healthy, because we consider that a normal dose of vaccine given to a child with a latent or slight infection can disturb the natural powers of resistance to such an extent that the infection in question will break out earlier or in more aggravated form. This we have shown clinically (1, 8, 9).

The vaccine treatment of animals with chronic infection thus shows that polyvalent and univalent vaccines have a definite effect which emerges in the aggravated course assumed by the disease. That the dose of vaccine (= antigen amount) plays a decisive part in this connection, that pre-infection vaccine treatment presumably increases the production of antibodies and thus partly protects the animals against later stress effect, and that a strongly aggravating effect on the disease is obtained when the stress is applied during the incubation period.

In our experiments we challenged the animals with varying amounts of tubercle bacilli (0.3, 0.1 and 0.05 mg). It was found that a small dose even can suffice to induce severe infection when the resistance of the host organism has been reduced because the antigen administered engages its antibodies.

The infection of chronic type (tuberculosis) we used has its given course and requires the organism to produce antibodies. The injection of vaccines containing antigens foreign to the organism affects the course adversely. This has also been shown by Packalén (6) in his studies of the effect of *E. coli* filtrate and staphylolysin on experimental guinea-pig tuberculosis.

In experimental animals whose antibody formation has already been engaged by a chronic infection we have bound more antibodies still by administering more heterologic antigen and have thus weakened the host's resistance to the infection it was trying to control.

Najjar & Fisher (1955) (5) have recently shown that up to 12 antibodies can be added to an antigen. In other words, several antibodies bind several antigens. In our work, increasing the number of antigens required of the host organism increased response in the form of antibody formation. But at the same time it bound antibodies and thus reduced the host's resistance to the infection.

It may be pointed out that there can be no question here of a Schwarzmans's phenomenon: we saw no local reactions at the site of the injection either in the present or in our earlier invest-

igations (13). The importance of observing the weight curves does not seem very great, at least not in this type of investigations. In a couple of experiments (10 and Experiment I of the present study) a distinct difference was observed in the weight curves between the groups treated with vaccine and the control groups: in the other experiments no difference was recorded.

Our experience to date, to which we shall add later, leads us to conclude that the course of chronic infections in experimental animals can be influenced with polyvalent or univalent vaccines. The present work is a link in the topical hostparasite relationship studies that figure in the experimental infection research of today.

#### SUMMARY

An investigation divided into four experiments, and described in two parts, comprising 85 guinea-pigs, was carried out to study the influence of heterologous vaccines on the resistance to a tuberculous infection. The experiments show that both polyvalent and univalent vaccines have a definite stress effect on the animals infected with  $H_{37}Rv$ -bacilli, resulting in more marked tuberculous lesions. The dose of vaccine, the antigen amount, also plays a part. Exposing the animals to stress with vaccines before the challenge seemed to influence the resistance less than if the vaccine treatment was given after the challenge only.

Vaccines are thus usable as stress factors in experimental host-parasite relationship studies. The authors explain the effect as dependent on the increase of antigens calling forth a maximum of antibodies and thus weakening the resistance to another heterologous infection present or in an incubation stage.

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## FEBRILE PHARYNGITIS AND CONJUNCTIVITIS

### AN EPIDEMIC ASSOCIATED WITH APC VIRUS INFECTION

by  
PER FORSELL, KAISA LAPINLEIMU, HELENA STRANDSTRÖM  
and NILS OKER-BLOM

(Received for publication April 11, 1956)

In August and September of 1955 there was an epidemic outbreak in Helsinki of an in this country hitherto unrecognised disease in children. The swimming pools of the city seemed to play an important rôle in the spread of the epidemic, and from some of the 57 patients treated at the hospital viruses of the APC type 4 were isolated. The disease thus seems to represent an epidemic of the Pharyngoconjunctival Fever recently described by Bell and others (1).

The group of Adenoidal-Pharyngeal-Conjunctival (APC) viruses (1, 4, 10) have attracted much attention during the last year and some other cytopathic agents isolated from patients with respiratory illnesses (2, 3, 6) have recently been shown to belong to this group (4). Besides in the U.S.A. agents of this type have so far been isolated at least in Sweden and England (5, 11, 12) and, according to a recent report, also in Canada (9). There are, however, only few epidemics described (1, 9, 10), so a brief account of the Helsinki epidemic may be of interest.

#### GENERAL ASPECTS

Several hundred cases very similar to those described in this paper occurred in the city during August and September. Most of them however, were treated at home by medical practitioners



and it is therefore impossible to get reliable figures of the actual number of cases.

The material reported on here consisted of 57 patients treated in the Aurora Hospital between the 10th of August and the 24th of September. All the patients were children between 3 and 15 years of age, and 44 of them were between 7 and 11 years of age. Two thirds of the patients were boys. Most of the cases occurred during the latter half of August.

#### CLINICAL FEATURES

The most characteristic symptoms were fever, pharyngitis and slight enlargement of the submaxillary lymph nodes. Most of the patients had a slight conjunctivitis, and in 7 cases a marked redness and swelling of the conjunctiva accompanied by a viscous purulent exudation was seen. No corneal opacities were observed. Both unilateral and bilateral conjunctivitis was observed.

Most patients had temperatures of 39° C to 40° C for several days. Fever persisted for 5 to 14 days, on the average for 7 to 8 days. The clinical course of one typical case is presented in Figure 1.

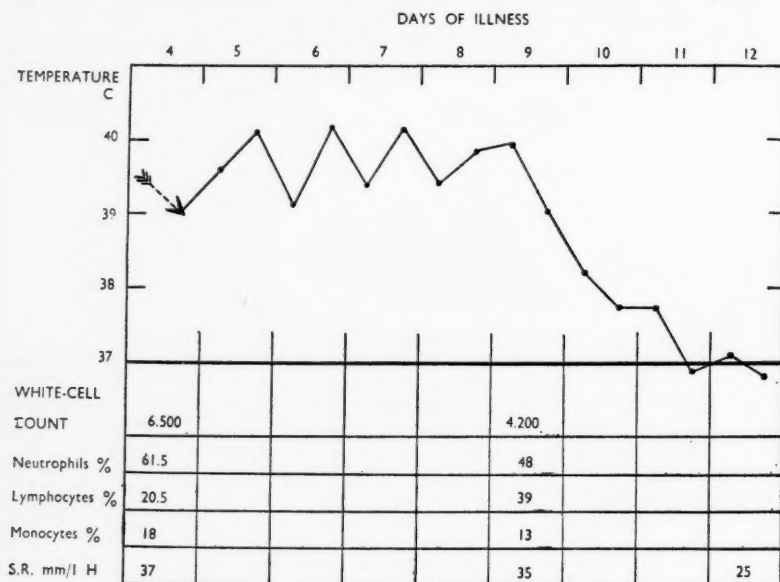


Fig. 1. — Clinical Course in Case 57 (T. J. Journ. No 2456/—55)

Besides the above mentioned symptoms, the most common symptoms were the following:

	Number of Cases
Rhinitis .....	36
Headache .....	36
Vomiting .....	38
Pains at swallowing .....	14
Diarrhoea .....	29
Abdominal pains .....	17
Pains in the back and legs .....	6
Stiffness of the neck and/or back .....	13

In 7 cases the stiffness of the neck was severe enough to indicate a lumbar puncture, but no increase in either the cell count or the amount of protein could be observed.

Antibiotics and sulfonamides seemed to have no influence on the clinical course of the several patients who received them either before admission or during their stay in the hospital.

As complications, sinusitis was seen in 11 cases, sinusitis and purulent otitis media in 2 cases and pneumonia in 1 case.

All the patients recovered and no permanent sequelae were observed.

#### LABORATORY FINDINGS

Total white cell counts showed normal values or a slight leukopenia. In 19 cases the counts were less than 6000 and in 5 of these less than 4000. Only in 4 of the cases complicated with a sinusitis were counts of 15,000 to 27,000 seen. The differential counts usually showed a relative granulocytosis.

The sedimentation rate (SR) was usually elevated being higher than 30 mm/l hour in 31 of the cases. Only in 3 cases was the SR less than 10 mm/l hour. All the patients showing complications had elevated SR values, but elevated values were seen also in patients free of complications.

No beta-hemolytic streptococci were isolated from the nose or throat in any case. No *Salmonellae* or other pathogenic enteric bacteria could be isolated. No rise in the antistreptolysin o-titer or the Widal-titer was observed.

## VIROLOGICAL FINDINGS

Throat washings and/or stools from 26 out of the 57 patients were investigated for the presence of virus in tissue culture by using human fibroblasts in roller tubes and HeLa cells according to methods previously described (7). From some of the patients acute phase and convalescent sera were tested for the presence of neutralizing antibodies against their own virus and against 6 prototype Adenoidal-Pharyngeal-Conjunctival viruses kindly supplied by Dr. Arne Svedmyr of the Central Municipal Bacteriological Laboratory, Stockholm, Sweden.

From 16 out of the 24 throat washings and from 21 out of the 26 stool specimens tested, agents cytopathogenic both for human fibroblasts and for HeLa cells were isolated. The cytopathic changes produced in HeLa cells were very similar to those produced by the prototype APC viruses. These agents were not pathogenic for infant mice and they could not be propagated on the CAM of embryonated chicken eggs. The neutralization tests performed on some of the paired sera with the patients' own strain of virus and with the prototype viruses showed in several cases a marked increase of neutralizing antibodies in the convalescent serum (Table 1). These neutralization tests also showed that the isolated

TABLE 1

TITERS OF THE PROTO TYPE APC VIRUSES AND THE PATIENTS OWN VIRUSES IN ACUTE PHASE AND CONVALESCENT SERA OF THREE PATIENTS WITH FEBRILE PHARYNGITIS AND CONJUNCTIVITIS

Virus Type	Serum					
	K.V.		S.H.		R.K.	
	Ac.	Conv.	Ac.	Conv.	Ac.	Conv.
1	256	256	<4	<4	<4	<4
2	<4	<4	64	256	256	320
3	<4	<4	<4	4	<4	<4
4	<4	64	<4	128	<4	64
5	<4	<4	16	16	<4	<4
6	<4	<4	<4	4	<4	<4
K.V.	<4	256	<4	256	<4	ND
S.H.	<4	256	<4	256	<4	ND
R.K.	<4	256	<4	256	<4	256

viruses were identical with or at least antigenically closely related to the Type 4 prototype virus.

A more detailed report on the agents isolated in connexion with this epidemic will be published elsewhere (8).

#### DISCUSSION

The epidemic described here shows clinically many similarities with some recently described outbreaks of Pharyngoconjunctival Fever (1). Some differences in the symptomatology of the disease, however, can be observed. Thus severe headache, gastrointestinal symptoms and stiffness of the neck or back occurred in a considerable number of the patients, and sinusitis was a fairly common complication.

The isolation of APC viruses from several of the patients and the demonstration of an increase in neutralizing antibodies against this virus in some of the cases seems, however, to establish a causal relationship between the clinical disease and an infection with this group of viruses. The fact that all the cases, including those from which no attempts were made to isolate virus, were clinically very similar speaks for the assumption that also most of these cases were caused by the same virus. Some support for this assumption comes from the fact that it was impossible to find any criteria suggesting a bacterial or leptospiral etiology and attempts to isolate other viruses, including Coxsackie viruses, were unsuccessful.

All the cases reported on here were children. No illness clinically similar to this has so far been diagnosed in adults in this hospital. It is noteworthy that nearly all the patients had been using the public swimming pools in the city before they fell ill, and as soon as the weather became colder and the swimming pools were closed the epidemic ceased. In this respect, too, the epidemic shows similarities with previous epidemics (1, 9, 10). Of the patients reported on here only two were brothers, and with this single exception there had been no other familial cases.

#### SUMMARY AND CONCLUSION

An epidemic of febrile pharyngitis apparently associated with infection with the Adenoidal-Pharyngeal-Conjunctival viruses occurred in Helsinki in August and September of 1955.

The main symptoms of the 57 patients treated in the hospital were fever, pharyngitis, submaxillary adenopathy, malaise, muscle pain and unilateral or bilateral conjunctivitis.

A considerable number of the patients, however, also showed gastrointestinal disorders, severe headache and stiffness of the neck.

The chief complication was sinusitis maxillaris occurring in about 20 per cent of the cases.

The disease spread primarily via swimming pools and transmission of the disease in the patients' homes was rare.

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## EFFECT OF COXSACKIE VIRUS ON THE GROWTH OF THE ROUS SARCOMA IN EMBRYONATED CHICKEN EGGS<sup>1)</sup>

### PRELIMINARY REPORT

by

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The work presented in this paper actually originated in 1950 in Dr. F. Duran-Reynals Laboratory, Yale University, New Haven, Conn., U.S.A. The intention was to study the effect of certain viruses on fowl tumors and especially on the Rous sarcoma in embryonated chicken eggs. For practical reasons, however, the work had to be interrupted at that time and has since been continued in this laboratory.

In the mean time a great amount of knowledge regarding the oncolytic effect of several viruses both *in vivo* and *in vitro* has accumulated. The literature up to 1954 has been summarized in a review by Moore (3). As far as the chicken tumors are concerned, it is known that several viruses have an oncolytic effect on the RPL 12 lymphoma in chickens (10). The most effective of the viruses tested was the Russian Spring-Summer-Encephalitis virus which also multiplied in the tumor cells.

It has recently been shown, however, that besides the clear oncolytic effect that viruses have on the tumors there are also virus-tumor systems in which the virus interferes with tumor growth without showing any oncolytic effect. It has been proposed in explanation of the latter phenomenon that there is a

<sup>1)</sup> This investigation was aided by a grant from the Sigrid Juselius Foundation.

competition between the virus and the tumor for some metabolite or nutrient required for the growth of both (1,11). In the case of the Cocksackie virus and the Rous sarcoma Cocksackie virus also seems to interfere with tumor growth without any demonstrable oncolytic effect. This paper and a subsequent report (7) therefore describe some preliminary results obtained with this virus-tumor system.

#### MATERIAL AND METHODS

*Tumor.* — The Rous sarcoma used in these experiments was obtained from Dr. F. Duran-Reynals in 1950 and has since then been kept in this laboratory in alternate embryonated egg and chicken passages according to methods described earlier (4).

*Tumor Titration.* — Titrations of the tumor-producing capacity of the sarcomas grown on the chorio-allantoic membrane (CAM) of embryonated chicken eggs were performed by injecting 0.25 ml of ten-fold suspensions of the tumor in to the right breast muscle of 10–14 day-old Leghorn chickens. For each dilution 3 to 6 chickens were used.

*Cocksackie Virus.* — The strain of Cocksackie virus used was a group A type 10 strain isolated in this country in 1950 (6). The virus was propagated in suckling mice 2–5 days of age. For the experiments with the Rous sarcoma the Cocksackie virus was partially purified with Bentonite (5).

*Cocksackie Virus Titrations.* — Material for the test was prepared by grinding the tissue in a mortar with sterile sand. Phosphate buffer, pH 7.2, was added to make a ten per cent suspension. The suspension was centrifuged in a PR 1 refrigerated centrifuge at 15,000 rpm for 15 minutes and the supernatant collected.

Ten-fold dilutions of the supernatant were prepared in the same buffer solution (the first dilution  $10^{-1}$  corresponding to a 10 per cent suspension of the tissue tested), and 0.03 ml of each dilution was injected intramuscularly into one litter of 2–4 day-old suckling mice, each litter containing 6–12 mice.

The  $LD_{50}$  for both the Rous sarcoma and the titrations with Cocksackie virus was calculated according to Reed and Muench.

*Normal Mouse Tissue.* — Suspensions of normal mouse muscle tissue were prepared from 5–7 day-old suckling mice in the



same manner as the Cocksackie virus suspension and partially purified with Bentonite.

*Inoculation of Embryonated Chicken Eggs.* — Eggs from Leghorn chickens were used, incubated for 11 days before inoculation. The material to be tested was inoculated onto the CAM according to a technic described earlier (4) and the eggs were then incubated for a further 8-day period.

#### EXPERIMENTAL

A 40 per cent suspension of Rous sarcoma grown for 8 days on the CAM of 11 day-old embryonated chicken eggs was mixed with equal parts of Bentonite-purified Cocksackie virus in the dilutions  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ . Each Cocksackie virus-Rous sarcoma mixture was immediately inoculated onto the CAM of 10–20 eggs in amounts of 0.05 ml, and the eggs were incubated for a further 8 days.

Simultaneously the same suspension of the Rous sarcoma was mixed with Bentonite-purified mouse muscle suspension in the same concentration as the Cocksackie virus suspension and inoculated onto the CAM in the same manner as the Cocksackie virus-Rous sarcoma suspensions.

TABLE 1

SIZE OF TUMORS ON THE CAM OF EMBRYONATED CHICKEN EGGS INOCULATED WITH ROUS SARCOMA ALONE AND ROUS SARCOMA TOGETHER WITH DIFFERENT CONCENTRATIONS OF COXSACKIE VIRUS AND NORMAL MOUSE MUSCLE TISSUE RESPECTIVELY.

Material	Days After Inoculation					
	4		6		8	
	No <sup>1</sup>	TMW <sup>2</sup>	No	TMW	No	TMW
RS-control .....	3	279	4	455	3	1407
RS and CV $10^{-1}$ .....	3	139	4	491	3	520
RS and CV $10^{-2}$ .....	4	83	4	171	7	695
RS and CV $10^{-3}$ .....	4	224	8	237	10	907
RS and NM $10^{-1}$ .....	4	129	3	962	6	1405
RS and NM $10^{-2}$ .....	6	175	6	552	8	1373
RS and NM $10^{-3}$ .....	3	142	5	535	7	1226

RS = Rous sarcoma CV = Cocksackie virus NM = Normal mouse tissue

<sup>1</sup>) Number of eggs in group <sup>2</sup>) Tumor mean weight

Finally, a 20 per cent suspension of the Rous sarcoma alone was inoculated onto the CAM of 10 eggs as a control.

Proof that the Coxsackie virus interferes with tumor growth was that the tumors obtained from eggs inoculated with the Coxsackie virus-Rous sarcoma suspensions were throughout

FIGURE 1

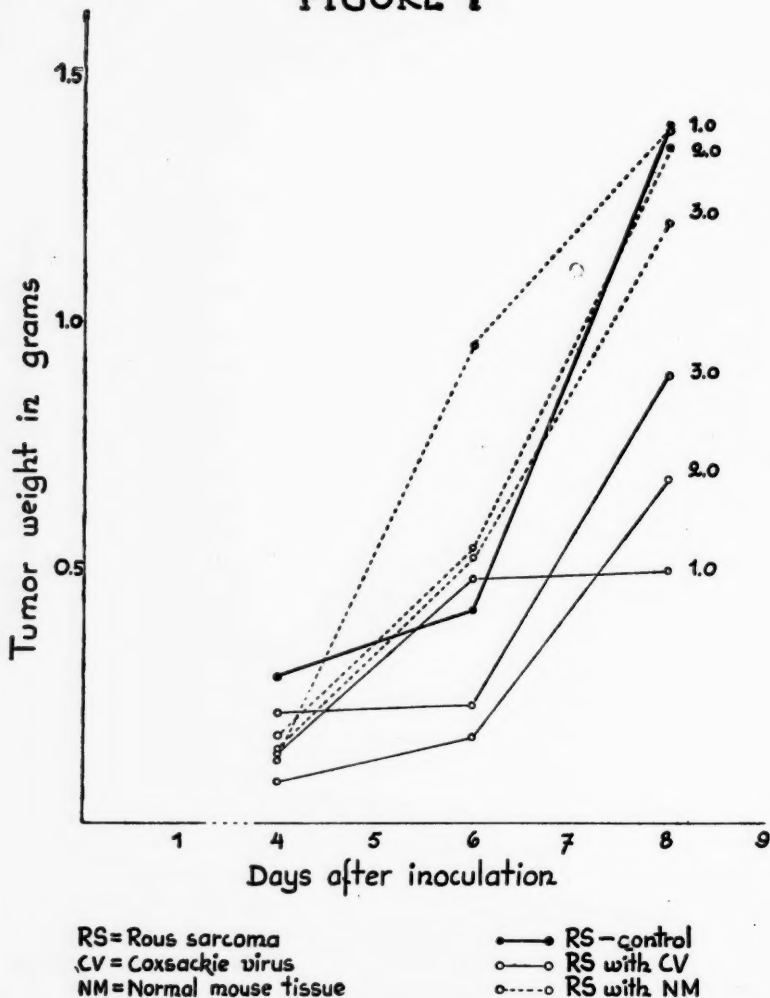


Figure 1. — Effect of Coxsackie virus (CV) and Normal mouse tissue (NM) on the growth of the Rous sarcoma (RS) on the CAM of embryonated chicken eggs. The numbers for the curves indicate the negative logarithms of the dilutions of CV and NM respectively.

smaller than either the controls or the tumors obtained from eggs inoculated with the normal mouse tissue — Rous sarcoma suspension. Tumor size also seems to depend on the concentration of Cocksackie virus used (Table 1, Figure 1).

Microscopically, no difference could be observed between the control tumors and tumors from eggs inoculated with Cocksackie virus or normal mouse tissue.

Although the Cocksackie virus thus seemed to interfere with the growth of the Rous sarcoma in embryonated eggs it did not seem to have any oncolytic effect and neither did it interfere with the transplantability of the tumors. This was evident from the titer of the tumor suspension as estimated in the chickens, which was about the same in all the groups tested including the control group. One of three chickens injected with a  $10^{-2}$  dilution (the lowest dilution tested) of sarcomas obtained from eggs inoculated with the mixture of Rous sarcoma and the 10 per cent suspension of Cocksackie virus survived without showing any tumor. With this single exception the tumors produced by the different dilutions both in this group and in the other groups tested were of the same size and type as the tumors in the control group.

The tumors obtained from eggs inoculated with Cocksackie virus

TABLE 2

INITIAL COXSACKIE VIRUS TITER IN SUSPENSIONS OF ROUS SARCOMA, IN SOLID TUMOR IN EMBRYONATED CHICKEN EGGS AND IN CAM OF EMBRYONATED CHICKEN EGGS AND TITER AFTER 8 DAYS OF INCUBATION.

Material	Cocksackie Virus Titer			
	Initial	After 8 Days Of Incubation		
		In Vitro at 37°C	In Solid Tumors In Eggs	In CAM Without Tumors
CV $10^{-1}$ .....	7.8	5.2		6.5
CV $10^{-2}$ .....	6.8			5.5
CV $10^{-3}$ .....	5.8			5.0
RS and CV $10^{-1}$ .....	7.9	6.6	6.1	
RS and CV $10^{-2}$ .....	6.6	4.7	5.7	
RS and CV $10^{-3}$ .....	N.D	4.5	5.4	

Titers are expressed as negative logarithms of dilutions of tissue

RS = Rous Sarcoma

CV = Cocksackie Virus

and Rous sarcoma also contained Cocksackie virus when harvested 8 days later and injected into chickens (Table 2).

The Cocksackie virus titers were, however, between 1 and 2 logs lower than in the initial inoculum. The Cocksackie virus titers of chorio-allantoic membranes inoculated with Cocksackie virus alone in the same concentrations showed about the same decrease in titer after 8 days of incubation. The same was true also of Cocksackie virus stored at 36° C in a 20 per cent suspension of Rous sarcoma. The titer of partially Bentonite-purified Cocksackie virus stored in the same manner for 8 days showed a decrease in titer of between 2 and 3 logs.

If the Cocksackie virus was inoculated onto the CAM before the Rous sarcoma the tumors obtained were still smaller, in comparison with the controls, than if the Cocksackie virus and the Rous sarcoma were inoculated simultaneously (Table 3).

TABLE 3  
SIZE AND TRANSPLANTABILITY OF ROUS SARCOMA GROWN IN EGGS PRE-TREATED  
WITH COXSACKIE VIRUS 2 DAYS PRIOR TO TUMOR INOCULATION.

First Passage In Pre-Treated Eggs			Tumors From First Passage In Untreated Eggs	
Pre-Treatment	Number Of Eggs	Mean Weight Of Tumors <sup>1)</sup>	Number Of Eggs	Mean Weight Of Tumors
Buffer Solution . . .	10	2.3	6	1.9
Cocksackie virus <sup>2)</sup> ..	10	0.6	6	2.0

<sup>1)</sup> Expressed in grams.

<sup>2)</sup> The LD<sub>50</sub> of the Cocksackie virus was 7.8 and it was inoculated as a 10 per cent suspension.

In this experiment no titrations were performed of either the Cocksackie virus or the Rous sarcoma, but the pre-treatment of the CAM with the Cocksackie virus did apparently not affect the transplantability of the tumors.

#### DISCUSSION

It has been pointed out in several connexions that anti-tumor action depends on multiplication of the virus (3). In these experiments the Cocksackie virus titers of chorio-allantoic membranes

both with and without Rous sarcomas were only somewhat higher than the titers of Cocksackie virus stored as such or in a suspension of Rous sarcoma. Considering, however, that the weight of the CAM or the tumors changed between about 100 and 1000 mg and that the first dilution ( $10^{-1}$ ) corresponded to a 1 : 10 dilution of the tissue resulting in a dilution of at least 1 : 50 to 1 : 500 of the initial inoculum, the titers were actually about 1 to 2 logs higher. It therefore seems reasonable to assume that there was some multiplication of the Cocksackie virus, particularly knowing that some strains of Cocksackie viruses multiply in embryonated chicken eggs (2, 8, 9). The experiments do not, however, explain whether there was a multiplication of the Cocksackie virus in the tumor cells themselves. In any case, no oncolytic effect of the Cocksackie virus could be demonstrated, any more than an effect on the transplantability of the tumors. This notwithstanding, the Cocksackie virus seemed to interfere with the growth of the tumors in the embryonated eggs.

Considering the fact that the Rous sarcoma is a virus-induced tumor, the assumption easily follows that the Cocksackie virus blocks the cells on the CAM thus rendering them or at least part of them less susceptible to the Rous sarcoma virus. The effect of the Cocksackie virus was greater if the virus was introduced onto the CAM before inoculation of the sarcoma, and it also seemed to depend on the amount of Cocksackie virus used. Another possibility would be that, like in the case of the MP virus and the Krebs 2 carcinoma (1,11), there is a competition between the two for some substance or substances in the embryonated egg.

Experiments to investigate these possibilities further have been initiated.

#### SUMMARY

A preliminary report is given on the interaction of Cocksackie virus and the Rous sarcoma in embryonated chicken eggs.

It was shown that the Cocksackie virus interfered with the growth of the Rous sarcoma on the chorio-allantoic membrane of embryonated chicken eggs without showing any oncolytic effect and without affecting the transplantability of the tumors.

The probable explanations to these findings were briefly discussed.

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## EFFECT OF COXSACKIE VIRUS ON THE GROWTH OF THE ROUS SARCOMA IN CHICKENS<sup>1)</sup>

### PRELIMINARY REPORT

by

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A preliminary report on the effect of Cocksackie virus on the growth of the Rous sarcoma in embryonated chicken eggs was presented in a previous paper (4). The conclusion reached was that Cocksackie virus interfered with tumor growth without causing any visible changes in the tumor cells and without affecting the transplantability of the tumors. It was assumed that the Cocksackie virus multiplied in the eggs and that the effect of the virus on the tumor could be ascribed either to a blocking of the cells in the CAM by the Cocksackie virus or to a competition between Cocksackie virus and the Rous sarcoma for some substance or substances in the eggs.

As mentioned above, the transplantability of the tumors to chickens was not affected although all the tumors initially infected with Cocksackie virus still contained virus when transplanted to chickens. Thus Cocksackie virus did not seem to have any effect on the growth of the Rous sarcoma in chickens, except probably in one chicken which survived the injection of a Rous sarcoma suspension containing Cocksackie virus in fairly high concentration.

It was of interest, however, to find out if Cocksackie virus administered prior to the inoculation of the Rous sarcoma would affect tumor growth in spite of the fact that the virus apparently does not multiply in chickens. Some preliminary results of this investigation are presented here.

<sup>1)</sup> Aided by grants from the Sigrid Juselius Foundation.



## MATERIALS AND METHODS

*Tumor.* — The Rous sarcoma used for the first experiment was obtained from an egg passage and for the second experiment from a previous chicken passage. The tumor was administered both as a cell suspension and as a cell-free filtrate. These were prepared according to methods described earlier (5). Of the cell suspension 0.25 ml and of the cell free filtrate 0.5 ml was injected into the right breast muscle of 1—2 weeks old Leghorn chickens.

Titration were made by injecting a ten-fold dilution of the material to be tested into 5—6 chickens in the above manner. The 50 per cent end point was calculated according to Reed and Muench.

*Coxsackie Virus.* — Besides the A10 strain of Coxsackie virus used for the previous experiments, an A4 strain obtained through the courtesy of Professor Sven Gard, The Institute for Virus Research, Karolinska Institutet, Stockholm, was also used for these experiments. The virus was purified and titrated in accordance with the methods given in the previous paper (4).

*Normal Mouse Tissue.* — Suspensions of normal mouse muscle tissue were also prepared according to methods described previously (4).

Coxsackie virus and normal mouse muscle were administered as 10 per cent suspensions either intravenously in the wing vein in amounts of 0.1 to 0.2 ml or intramuscularly in amounts of 0.5 ml.

## EXPERIMENTAL

*Experiment 1.* — Thirty-four five-day old chickens were divided into the following groups:

*Group 1.* This group, consisting of six chickens, was used as a control for Coxsackie virus. The chickens received intravenous injections of Coxsackie virus only. Altogether 4 injections of A10 and 2 injections of A4 were given at intervals of 2—7 days. One of these chickens was sacrificed immediately and another 18 days later for a study of the possible presence of virus in the chickens and the possible occurrence of antibodies against Coxsackie virus.

*Group 2.* — Six chickens were given Cocksackie virus A10 intravenously at 2-day intervals. Three days after the second Cocksackie virus injection the chickens were inoculated with a 20 per cent cell suspension of Rous sarcoma grown on the CAM of 11-day eggs for 7 days.

*Group 3.* — Five chickens were given the same pre-treatment as in group 2. Three days after the second injection they also were inoculated with the same Rous sarcoma suspension, but in this group the chickens received four additional Cocksackie virus injections intravenously at the same time as the Cocksackie virus controls in group 1. Cocksackie virus of type A10 was given twice and Cocksackie virus of type A4 twice.

*Group 4.* — The six chickens in this group were inoculated with Rous sarcoma without receiving any pre-treatment. But simultaneously with the Rous sarcoma inoculation they received Cocksackie virus A10 intravenously, and thereafter they were treated with intravenous Cocksackie virus injections like the chickens in group 3.

*Group 5.* — At the same time as the chickens in the other groups received the inoculation of Rous sarcoma the six chickens in this group were injected with a suspension consisting of equal parts of a 40 per cent suspension of the Rous sarcoma and a 10 per cent suspension of Cocksackie virus A10. They had received no pre-treatment and were not treated after the tumor inoculation.

*Group 6.* — This group consisting of 5 chickens, served as tumor controls, receiving nothing but a 20 per cent suspension of the Rous sarcoma.

The results of these experiments are presented in Table 1.

TABLE 1  
EFFECT OF COXSACKIE VIRUS ON THE GROWTH OF ROUS SARCOMA IN CHICKENS  
(EXPERIMENT 1)

Group	Number Of Chickens	Length Of Life In Days	Mean Length Of Life In Days	Deviation From Controls	
				Days	Per Cent
2 <sup>1)</sup>	6	14—20	17.2	+ 3.2	23
3	5	17—22	18.2	+ 4.2	30
4	6	10—20	13.3	— 0.7	
5	6	12—17	13.7	— 0.3	
6	5	12—15	14.0		

<sup>1)</sup> For explanation of groups, see text, Experiment 1.

Of the chickens receiving only Cocksackie virus, two were sacrificed during the experiment for virus and antibody studies. Of the remaining four chickens three were sacrificed 4 months later without having showed any pathologic lesions. (One chicken died 49 days after the initiation of the experiment, apparently of an intercurrent infection.) No virus could be isolated from the muscle, liver or spleen of the chickens sacrificed during the experiments, and no increase in neutralising Cocksackie antibodies could be demonstrated. Thus the Cocksackie virus injections did not seem to affect the chickens and no evidence was obtained of multiplication of the Cocksackie virus in the chickens.

In spite of this, Cocksackie virus given prior to tumor inoculation did seem to interfere with tumor growth. This was suggested by the fact that among the chickens receiving Rous sarcoma, groups 4, 5 and 6 showed about the same survival time, i.e. 13.3 to 14.0 days, whereas the chickens in groups 2 and 3, viz. those pre-treated with Cocksackie virus, had survival times of 17.2 to 18.2 days. The size of the tumors, the frequency of metastases and the hemorrhagic lesions in the chickens in the different groups, however, showed about the same variation, and thus the only observable difference was the somewhat prolonged survival time of the chickens pre-treated with Cocksackie virus.

Attempts to isolate Cocksackie virus from the tumor, liver and spleen of one of the chickens in group 5 were unsuccessfull.

*Experiment 2.* — In the previous experiment the tumors grew very fast. It was therefore assumed that the differences in survival time would be more pronounced in chickens with tumors of slower growth. Such tumors were obtained by inoculating somewhat older chickens with a diluted cell-free filtrate of the Rous sarcoma (2).

According to the previous experiment, Cocksackie virus injected simultaneously with or after the tumor inoculation had no effect whatsoever on the growth of the tumors. Only if the chickens were pre-treated with Cocksackie virus could some effect be obtained. Therefore the chickens in this experiment were given two intravenous injections of Cocksackie virus before they were inoculated with the Rous sarcoma virus. Some of the chickens were also injected intramuscularly with Cocksackie virus in the same breast that later received the Rous sarcoma virus in-

oculation. Since it was impossible to exclude the chance that the effect arose simply from the introduction of foreign material the same number of chickens were injected with normal mouse muscle tissue in the same manner. The experiment thus consisted of the following groups:

*Group 1.* — Control chickens receiving 0.5 ml of about 100 tumor-producing doses of a cell-free filtrate of the Rous sarcoma obtained from a previous chicken passage.

*Group 2.* — Chickens which prior to the inoculation of 100 tumor-producing doses of the cell-free filtrate, had received two intravenous injections of Cocksackie virus A10 with an interval of 7 days. The Rous virus was given on the day of the second Cocksackie virus injection.

*Group 3.* — This group was injected with Cocksackie virus intramuscularly twice before the injection of the Rous sarcoma virus. The injections were given in the same breast as the tumor virus and the tumor virus, as with group 2, was given on the day of the second Cocksackie virus injection.

*Group 4.* — This group was exactly similar to group 2 except that normal mouse muscle tissue was used instead of Cocksackie virus.

*Group 5.* — These chickens were treated like the chickens in group 3, but instead of Cocksackie virus the chickens received intramuscular injections of normal mouse muscle tissue.

As in the first experiment, the pre-treatment with Cocksackie virus here, too, resulted in a prolongation of the survival time, and in some of the chickens no tumors at all were produced. The

TABLE 2

EFFECT OF COXSACKIE VIRUS AND NORMAL MOUSE MUSCLE TISSUE ON THE GROWTH OF ROUS SARCOMA IN CHICKENS (EXPERIMENT 2)

Group	Number Of Chickens	Length Of Life In Days	Mean Of Length Of Life In Days	Deviation From Controls		Number Survived	Survival In Per Cent
				Days	Per Cent		
1 <sup>1)</sup>	10	21—39	30			0	0
2	15	25—63	41	+ 11	36	3	20
3	15	30—76	44	+ 14	47	6	40
4	14	26—56	36	+ 6	20	1	7
5	18	31—63	38	+ 8	27	1	6

<sup>1)</sup> For explanation of groups, see text, Experiment 2.

most marked effect was obtained in the group which had received Cocksackie virus intramuscularly. (Table 2)

The survival time among chickens developing tumors was 47 per cent longer than in the control group, and 6 out of 15 chickens, i.e. 40 per cent, were sacrificed 130 days later without having grown any tumors at all. The same tendency was also seen, however, in the groups injected with normal mouse muscle tissue, although the prolongation of the survival time of chickens developing tumors was not as pronounced as in the Cocksackie virus groups. The number of tumor-free surviving chickens was also smaller than in the groups receiving Cocksackie virus.

With one exception the type of tumor, the distribution of metastases and the hemorrhagic lesions were very similar in all the groups. The exception was a chicken in group 4, the group which received normal mouse muscle tissue intramuscularly; this chicken developed a tumor about three months after inoculation. The chicken was in good condition and the tumor found was a firm encapsulated tumor of chicken egg size. Attempts to transplant the tumor to embryonated chicken eggs were unsuccessful, as were attempts to transplant the tumor to chickens via cell-free filtrate. Transplanted as a cell suspension to 10 day-old chickens, however, it produced typical Rous sarcomas in the chickens.

A circumstance probably worth mentioning is that, because of change of staff in the animal room, the number rings of the growing chickens were not enlarged for about one week and the result was oedema and ulceration of the right leg. These chickens became lean and were in poorer general condition until the rings were changed. Seven out of the 11 chickens surviving without tumors belonged to this group. It has to be remembered, however, that at that time they had already survived for 69 days without showing any tumors. This was in contrast to the controls all of which had died at least one month earlier.

The chickens were not weighed, but with these exceptions all the chickens seemed to be in good condition throughout the experiment until they developed tumors.

#### DISCUSSION

The experiments presented in the previous paper showed that Cocksackie virus interfered with the growth of the Rous sarcoma

on the CAM of embryonated chicken eggs, and some evidence was obtained of the multiplication of the Cocksackie virus in the eggs. No inhibition of tumor growth was obtained with normal mouse tissue and thus the anti-tumor action can apparently be ascribed to some mechanism connected with the multiplication of the Cocksackie virus in the eggs.

In the experiments presented in this paper no evidence could be found of the multiplication of Cocksackie virus in the chickens or in the tumors produced in them. If, as has been stated (3,6), the anti-tumor action of viruses depends on the multiplication of the virus in the host or in the tumor, the effect of Cocksackie virus on the growth of the Rous sarcoma in chickens cannot be compared to the virus-tumor systems, especially considering the fact that a similar although less pronounced effect was obtained with normal mouse muscle tissue. Some other plausible explanation has therefore to be found. It is, for instance, known that starvation of the host has an influence on the growth of the Krebs 2 carcinoma in mice (1,7). It is possible that the pre-treatment of the chickens used in these experiments affected the general condition of the animals, resulting in decreased susceptibility to the tumor. The intramuscular injections may have had a similar local effect.

Further experiments are required, however, before the interaction of Cocksackie virus and the Rous sarcoma can be interpreted.

#### SUMMARY

Intravenous or intramuscular injections of Cocksackie virus into chickens prior to the inoculation of cell suspensions or cell-free filtrates of the Rous sarcoma were shown to retard the growth of the tumor. In some cases this pre-treatment of the chickens totally inhibited tumor growth. A similar but less pronounced effect was obtained with the injection of normal mouse muscle tissue prior to tumor inoculation.

These findings were discussed in the light of the anti-tumor action obtained with the Cocksackie virus in embryonated chicken eggs, and some probable explanations were suggested.

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## KUMLINGE DISEASE

A MENINGO-ENCEPHALITIS OCCURRING IN THE AALAND ISLANDS<sup>1)</sup>

by

N. OKER-BLOM

(Received for publication April 11, 1956)

On Kumlinge, one of the bigger islands in the Aaland archipelago, several cases of meningo-encephalitis have occurred since 1942 and during the last few years some cases have been observed also in other parts of the archipelago. An investigation to elucidate the etiology of the disease was therefore initiated in the summer of 1954. The purpose of this paper is to give a brief survey of the clinical picture of the disease and a preliminary report on some of the etiological studies so far performed.

### GENERAL ASPECTS

The archipelago, and within it the islands of Kumlinge, will be clear from Figure 1. The population of the whole archipelago is 22,185, of Kumlinge 788. On Kumlinge and the nearest islands belonging to the same district altogether 39 cases of a disease characterized by symptoms of the central nervous system and called locally »Kumlinge disease» have been observed for the period 1942 to 1955. There was an accumulation of cases in 1950 (Table 1), the total number being 11.

<sup>1)</sup> Aided by grants from the Sigrid Juselius Foundation and Samfundet Folkhälsan i Svenska Finland.

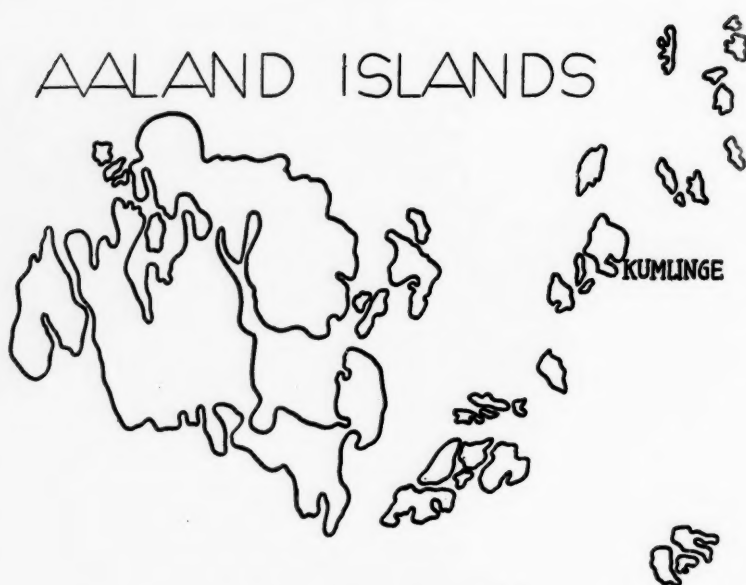


Fig. 1. — Map of the Aaland Archipelago.

All the cases occurred during the summer and early autumn with a peak in August and September. There were twice as many females as males affected; the mean age of the patients was 36 years. No cases occurred in children below 10 years of age. Two of the 39 cases ended fatally.

TABLE 1

DISTRIBUTION OF CASES IN KUMLINGE ACCORDING TO YEAR, SEASON, SEX AND AGE

Year	1942	1943	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954
	2	—	2	3	7	6	2	2	11	1	1	—	—
Month		I	I	III	IV	V	VI	VII	VIII	IX	X	XI	XII
	—	—	—	—	—	—	2	4	11	15	7	—	—
	Total	♀	♂	1—10	11—20	21—30	31—40	41—50	51—				
	39	26	13	—	6	8	8	12	5				

Most of the patients were treated at the cottage hospital and only a few were admitted to the provincial hospital in Åbo. One of the patients from another part of the archipelago was admitted to the Maria Hospital in Helsingfors.

## CLINICAL FEATURES

The 39 patients from Kumlinge were discharged from the hospitals with the diagnoses listed in Table 2.

TABLE 2

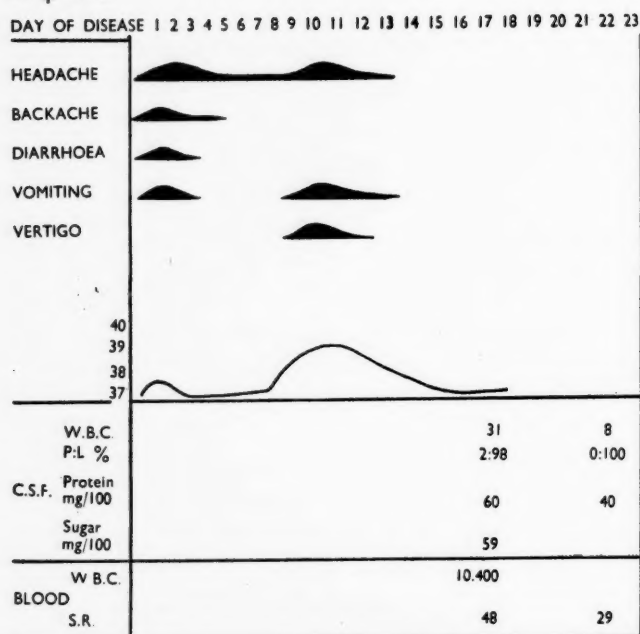
DISTRIBUTION OF THE CASES IN KUMLINGE ACCORDING TO CLINICAL DIAGNOSIS

Diagnosis	Number
Infectio acuta. Cephalalgia .....	2
Infectio acuta. Meningismus .....	2
Infectio acuta. Meningitis serosa .....	4
Meningitis serosa .....	21
Meningitis serosa. Paratyphus B?.....	3
Meningitis serosa. Poliomyelitis non-paral. ....	1
Meningitis chr. nonspec. ....	1
Poliomyelitis ant.ac. ....	2
Encephalitis .....	3
Total .....	39

In the three cases where paratyphoid was suspected the bacteriological and serological findings did not support the salmonella diagnosis. Of the two patients given a diagnosis of paralytic poliomyelitis, one had a slight paralysis of the muscle of the right thumb. The other patient was a 66 years old woman who after a short severe illness succumbed to the syndrome of Landrys paralysis.

Typical of most of the cases was a diphasic type of illness: there was an initial febrile period followed by apparent recovery, and then a new attack of fever accompanied by symptoms of the central nervous system (Figures 2 and 3).

S.L. ♀ 42 YRS



H.T. ♂ 44 YRS

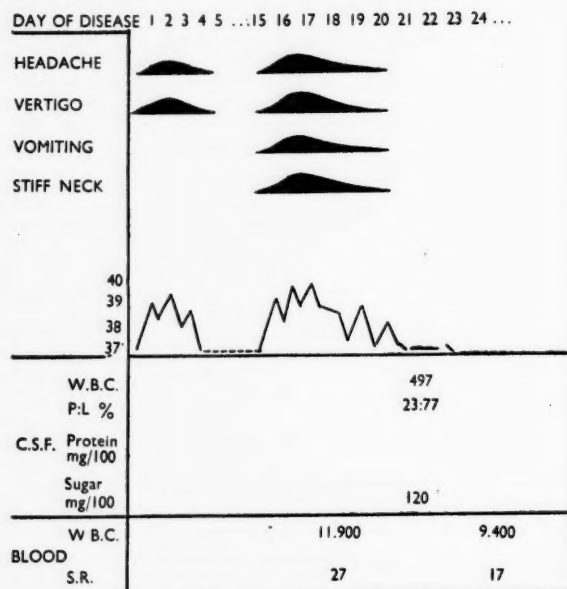


Fig. 2 and 3. — Clinical course in two typical cases of the Kumlinge disease.

The most common symptoms and clinical findings for 22 of the Kumlinge patients are listed in Table 3.

TABLE 3

SYMPTOMS AND PHYSICAL SIGNS OF ILLNESS IN 22 PATIENTS WITH THE CLINICAL SYNDROME OF THE KUMLINGE DISEASE

Diphasic illness .....	19
Latency period .....	3 days — 19 days (mean 12 days)
Elevated temperature during II phase .....	2 days — 7 days (mean 5 days)
Headache .....	21
Vomiting .....	17
Stiff neck .....	18
Positive Lasegue sign .....	7
Positive Kernig sign .....	5
Paralyses .....	2
Herpes labialis .....	1
Duration of illness .....	17 days — 38 days (mean 25 days)
Treated in hospital .....	5 days — 20 days (mean 11 days)

It was not possible at the cottage hospital to perform white blood cell counts or to analyse the cerebrospinal fluid and therefore such data have been obtained only for those few patients treated in the central hospitals. In these patients, the white blood cell counts showed a slight leukocytosis, slightly elevated values for the sedimentation rate were seen and in the cerebrospinal fluid there was a lymphocytosis with counts of 20 to 500 cells per cu.mm. The protein in the CSF was normal or slightly elevated and the same was true of the sugar.

#### ETIOLOGICAL STUDIES

Blood specimens were collected from 26 of the patients in the summer of 1954. No blood specimens could be obtained from the rest of the patients on Kumlinge either because they had moved and could not be reached or they were absent at the time the specimens were collected. Three patients from other parts of the Aaland islands who had had similar symptoms gave blood specimens in the autumn of 1955 and in January 1956. The time which had elapsed between the illness and the collection of the blood specimens was thus between three weeks and 12 years.

All the sera, including some sera from healthy persons on Kumlinge, were tested for agglutinating antibodies against *Leptospira*. These sera were tested for antibodies against the three types of poliomyelitis virus as well, and some of the sera, including sera of some healthy adults and children from other parts of the country, were tested for antibodies against Louping Ill virus.

The *Leptospira* investigations were kindly performed by Dr. A. Salminen at the Department of Serology and Bacteriology, Helsinki University, according to methods described by him elsewhere (10).

The poliomyelitis antibody studies were performed in HeLa cell cultures as described in previous publications (6, 7).

The Louping Ill virus used for these studies was kindly supplied by Dr. D. G. ff. Edward, The Wellcome Research Laboratories, Beckenham, Kent, England. Neutralization tests were performed in mice according to a method described elsewhere (5). The procedure was briefly as follows: Undiluted serum and serial ten-fold dilutions of virus were mixed and incubated at 36° C for 2 hours. The serum virus mixtures and ten-fold dilutions of the virus alone were then injected intracerebrally into albino mice weighing between 9 and 11 grams in groups of five. The 50 per cent endpoints were estimated according to Reed and Muench and the neutralization indices calculated. A similar neutralization test with a hyperimmune sheep serum supplied in 1951 was set up as a control. A method of estimating antibodies against Louping Ill virus in cultures of HeLa cells was worked out in this laboratory (5); some neutralization tests were simultaneously performed in tissue culture.

The serum of one of the persons who had had the disease showed an agglutination titer of 1:1000 against *Leptospira sejroe*. With this single exception all the sera tested were negative.

Sera from 21 of the 39 patients were tested for the presence of antibodies against the three types of poliomyelitis virus in a 1:4 dilution. Antibodies against type 1 were found in 12, against type 2 in 20 and against type 3 in 10 of the 21 sera tested.

The sera of all of the persons so far tested and representing typical cases of Kumlinge disease contained antibodies against the Louping Ill virus as estimated by neutralization tests both in mice and in tissue culture. (Table 4). On the other hand no neutralizing antibodies were demonstrated in the sera collected from healthy adults and children living in other parts of the country.

A few attempts to isolate by intracerebral inoculation of mice virus from ticks collected in autumn 1955 on Kumlinge proved unsuccessful.

TABLE 4

DISTRIBUTION OF ANTIBODIES AGAINST LOUPING III VIRUS AS ESTIMATED BY  
NEUTRALISATION TESTS IN MICE AND IN TISSUE CULTURE (STRAIN HELA CELLS)

		Antibodies against Loupin III Virus	
		Neutralisation Index estimated In Mice	Neutralisation Of 100 CPD 50 In HeLa Cells
Specific Immune Serum		6320	1 : 1600
Patients with the Kumlinge Disease	G.R. ....	632	
	B.N. ....	1250	+
	E.R. ....	500	
	A.H. ....	300	+
	A.E. ....	1250	+
	E.E. ....	63	+
	L.L. ....	320	+
	H.T. ....	4000	+
	N.N. ....	3750	+
	K.K. ....	6320	+
	E.L. ....		+
	O.S. ....		+
	H.K. ....		+
	H.E. ....		+
	H P. ....		+
Healthy adults and children	N.O. ....	0	
	I.R. ....	0	
	S.K. ....	0	
	M.O. ....	0	
	296 ....	10	—
	402 ....	< 10	—
	248 ....	10	—
	368 ....		—
	420 ....		—
	422 ....		—
	198 ....		—
	246 ....		—
	247 ....		—



## DISCUSSION

All the cases of the so-called Kumlinge disease described here seem to have many symptoms in common. The disease was usually diphasic; the second phase was accompanied by symptoms from the central nervous system classified as a meningo-encephalitis usually without paralyses.

Most of the cases occurred on the same small island and only during the last few years have similar cases occurred on other islands in the Aaland archipelago. The disease has thus had an endemic character.

As far as the etiology of the disease is concerned, it seems reasonable to exclude the possibility of a Leptospirosis because of all the sera obtained from patients who had had the disease only one showed antibodies against *Leptospira sejroe*.

An argument against the possibility of endemic poliomyelitis is the fact that no children at all were affected and that the incidence of paralyses was very low. In the two cases showing paralyses these were of a type which does not necessarily support a diagnosis of poliomyelitis. On the other hand the seasonal distribution of the cases was similar to that of poliomyelitis. It is noteworthy also that in 20 out of 21 of the persons who had the disease antibodies were stated against type 2 poliomyelitis virus. Only one of the patients, however, was under 20 years of age and according to a previous study concerned with the distribution of antibodies against the three types of poliomyelitis in this region the frequency of type 2 antibodies in this age group and for the whole archipelago was 83 per cent. During the severe type 1 poliomyelitis epidemic on the Aaland island in 1953 no cases occurred on Kumlinge. At the beginning of and during the epidemic no virus could be traced on Kumlinge and in the following year type 1 poliomyelitis virus was isolated from one person living on Kumlinge (6, 7).

The fact that all the cases on Kumlinge had occurred several years before this investigation was initiated of course excludes a virological diagnosis. In two similar cases occurring on the neighbouring islands in 1955, however, a virological diagnosis of poliomyelitis could not be verified.

For several reasons, therefore, the idea of endemically occurring

poliomyelitis was abandoned and another probable etiology was searched for.

Considering the close proximity of the islands to Russia, the occurrence of Russian spring summer encephalitis (11, 12, 13), the reports of the occurrence of tick-borne encephalitides in Eastern Europe (14, 17) and a recent report from Sweden on encephalitides belonging to this group (15) a relationship between Kumlinge disease and the above mentioned group of encephalitides seems likely. The symptoms of the disease are very similar particularly to those of the Central European Encephalomyelitis recently described (4, 16). It also shows great similarities to the infections of Louping Ill in man (2, 9) and in sheep (8).

The presence of antibodies against Louping Ill virus in the sera of persons who have had the Kumlinge disease speaks for the above assumption. In no case, however, was an increase in antibodies during the disease established, and very few »normal» sera have been tested. Because most of the patients had the disease many years ago a history of tick bites could be proved in a few cases only. No virus has been isolated and no attempt has so far been made to find natural infections in animals in the region.

In spite of all this, it seems probable, however, that Kumlinge disease belongs to the Russian spring summer encephalitis-Louping Ill group of encephalitides. In view of the close antigenic relationship between the viruses in this group (1, 3) the exact nature of the disease remains obscure. That the neutralization indices obtained with the Louping Ill virus were high, that sheep-farming is very common in the Aaland Islands, and finally that the tick *Ixodes ricinus* occurs in this region are all points that may well support the view that Kumlinge disease actually represents human infections with Louping Ill virus.

The data so far obtained are, however, by no means conclusive. Further studies are in progress.

#### SUMMARY

The so-called Kumlinge disease, representing a syndrome of meningo-encephalitis with a diphasic illness occurring endemically in the Aaland Islands, is described. Some preliminary studies have been made of the etiology of the disease. It is assumed that the

disease belongs to the Russian spring summer encephalitis-Louping Ill group of virus encephalitides. The possibility that the cases described represent a Louping Ill infection in man is discussed.

The author's thanks are due Dr. D. G. ff. Edward who kindly supplied the strain of Louping Ill virus used, to Dr. H. Cox and Dr. D. Wolfe for the supply of sheep immune serum, to Dr. O. Torckell and Dr. N. von Hellens for their collaboration in collecting the data of the patients, and to Dr. J. Wickström and Professor B. von Bonsdorff for placing the hospital journals at the disposal, to Miss M. Blom who assisted in the collection of the blood specimens, and finally to Miss I. Rouhia for her excellent assistance in the laboratory work.

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## DIAGNOSIS OF POLIOMYELITIS BY MEANS OF VIRUS ISOLATION AND NEUTRALISATION TESTS

WITH SOME REMARKS ON THE HETEROTYPIC ANTIBODY RESPONSE<sup>1)</sup>

by

N. OKER-BLOM, HELENA STRANDSTRÖM and KAISA LAPINLEIMU<sup>2)</sup>

(Received for publication April 20, 1956)

The value of the tissue culture technique for the diagnosis of poliomyelitis has recently been outlined by Enders (1). Some of the results obtained with this method especially during the epidemic of poliomyelitis in Helsinki in 1954 will be presented in this paper. The number of virus isolations from patients with paralytic poliomyelitis has, however, been fairly low compared to the results obtained elsewhere (1, 4, 7, 13) and some probable reasons for this have been searched for in the light of recent investigations. The neutralization tests performed on the acute phase and convalescent sera of the patients revealed that an increase in antibodies against more than one virus type was frequently seen; therefore this heterotypic antibody response (8, 12, 13) will also be briefly discussed.

### MATERIAL AND METHODS

The results presented here are based on specimens obtained from patients of the Aurora Hospital during the years 1953, 1954 and 1955. The years 1953 and 1955 were ordinary regarding the fre-

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<sup>2)</sup> With the technical assistance of Miss Vivi Perttunen Miss Karin Sandelin Sylvi Ahvenniemi and Rauni Malinen.

quency of poliomyelitis, but 1954 was an epidemic year. In 1953, stools from 36 patients were investigated. The corresponding numbers for 1954 were 184 and for 1955 105. The antibody studies referred to here have been performed on sera obtained mainly from the patients in 1954 and on a few from 1955 and thus 136 pairs of sera from 1954 and 13 from 1955 have been tested for neutralizing antibodies against the three types of poliomyelitis viruses.

The stool specimens were collected as early as possible in the course of the disease and stored in a deep freezer at  $-15^{\circ}\text{C}$  to  $-18^{\circ}\text{C}$ . They were tested within 3 to 625 days after collection.

The serum specimens were usually collected at the day of admission and 10 to 14 days later. Some times, however, the first specimen was not collected until four to five days after admission. The serum was separated by centrifugation and stored in a refrigerator at  $+4^{\circ}\text{C}$ .

*Virus Isolations and Typing.* — The main procedure was as follows: Ten per cent suspensions of the stool specimens were inoculated in amounts of 0.1 ml into three roller tubes of human embryonic fibroblasts. When cytopathic changes were seen a second passage in HeLa cell cultures was done, and from this second passage the typing was performed in HeLa cells. The details of the tissue culture technique used in this laboratory and of the methods for virus isolations and typing have been described at large in other connexions (5, 9, 10). The HeLa cells were kindly supplied by Dr. J. T. Syverton and Dr. W. F. Scherer, University of Minnesota, Minneapolis, Minn. U.S.A. The immune sera used were either monkey sera obtained through the courtesy of Dr. J. Melnick, Yale University New Haven, Conn., U.S.A. or guinea pig sera prepared in this laboratory according to the method of Gard and his associates (3). A pool of the three type sera was included to each typing.

*Neutralization tests.* — The sera were titrated in four fold dilutions beginning with a dilution of 1:4 against 100 CPD<sub>50</sub> of virus grown in HeLa cell cultures. The viruses used were a Brunhilde, the MEF 1 and the Saukett strains which were kindly supplied by Dr. Tore Wesslen at the State Bacteriological Laboratory, Stockholm, Sweden.

## RESULTS

Among the polio viruses isolated type 1 was predominant. Type 2 viruses were isolated in four cases and type 3 viruses in one case only in 1956. The isolation attempts in 1953 and 1955 are few compared to those in 1954, but in spite of this there seems to have been during the years an increase in the isolations of cytopathogenic agents not typable by poliomyelitis immune sera (table 1). Such agents were isolated both from paralytic and from non-paralytic cases.

TABLE 1

CORRELATION BETWEEN CLINICAL DIAGNOSES AND FECAL VIRUS ISOLATIONS IN 317 PATIENTS IN 1953, 1954 AND 1955.

Year	Diagnosis	Sub-jects	Number Of Isolations				Polio Virus Recoveries
			Poliomyelitis			Other Cyto-patho-genic Agents	
			1	2	3		
1953	Paralytic poliomyelitis ..	15	5	0	0	0	% 33
	Nonparalytic » ..	11	5	0	0	1	45
	Other diagnoses .....	10	0	0	0	0	0
1954	Paralytic poliomyelitis ..	116	64	1	0	11	56
	Nonparalytic » ..	60	14	0	0	13	23
	Other diagnoses .....	8	0	0	0	2	0
1955	Paralytic poliomyelitis ..	46	31	1	0	6	69
	Nonparalytic » ..	47	14	2	0	11	34
	Other diagnoses .....	12	0	0	0	2	0

In the following the attention will be mainly directed towards the specimens collected during the epidemic in 1954.

Only from part of the patients in 1954 acute phase and convalescent sera were obtained and from part of these patients only virus isolations were attempted.

Either on the basis of virus isolation or of an antibody increase the diagnosis of poliomyelitis could in these cases be virologically verified in 79 per cent of the paralytic cases only and in 50 per cent of the non-paralytic cases.

TABLE 2  
CORRELATION BETWEEN TYPE I ANTIBODY INCREASE AND ISOLATION OF  
POLIOMYELITIS VIRUS OF TYPE I

	Paralytic			Nonparalytic		
	Number Of Cases	Virus Iso- lated	Positive Per Cent	Number Of Cases	Virus Iso- lated	Positive Per Cent
Antibody increase observed .....	48	31	65	18	8	44
Antibody increase not observed .....	23	15	69	6	2	33

From table 2 it will be apparent that among the patients showing a clear increase in antibodies against type 1, which was the prevalent virus type during the epidemic, virus isolation failed in 35 per cent of the cases in the paralytic group and in 56 per cent of the cases in the non-paralytic group. On the other hand virus was recovered in 65 per cent of the paralytic cases showing no increase in antibodies and in 33 per cent of the corresponding non-paralytic group. In all the latter cases, however, the first serum already contained neutralizing antibodies in high titer.

These findings show that the virus recovery rate was far too low.

It is known that virus is more often isolated from children than from adults (4, 13). About half of the patients from whom

TABLE 3  
INFLUENCE OF AGE OF PATIENT, TIME OF STORAGE OF SPECIMENS AND SIZE OF  
INOCULUM ON THE RECOVERY RATE OF POLIOMYELITIS VIRUS FROM FECAL  
SPECIMENS IN PARALYTIC CASES

Inoculum	Age	Time Of Storage Of Specimens In Days						Total Positive	Positive %
		1—90		91—180		180—			
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.		
0.1 ml	< 1—10	8	1	7	4	6	7	21	64
	11—20		1	1	1	3	5	4	36
	21—		2	3	4	7	12	10	36
0.5 ml	< 1—10					9	4	24	73
	11—20					3	5	4	36
	21—					14	5	17	61



isolations were attempted were over 21 years of age. From table 3 it is apparent that the percentage of isolations also in this material was much higher in children below ten years of age. Several specimens were not collected until during the third week after the onset of symptoms and later. The virus recovery rate was somewhat higher among the specimens collected early in the disease (table 4).

TABLE 4  
CORRELATION BETWEEN RECOVERY OF POLIOMYELITIS VIRUS AND DURATION OF ILLNESS AT AT TIME OF FECAL SPECIMEN COLLECTION

Days Of Illness	Paralytic Poliomyelitis		Nonparalytic Poliomyelitis	
	Number Positive	Number Negative	Number Positive	Number Negative
1—7	21	12	3	11
8—14	17	7	5	10
15—21	6	3	1	3
22—	1	2		

The effect of the time of storage before the specimens were tested is shown in table 3. On the whole there were more positives among the specimens tested immediately or within three months of storage than among specimens stored for three to six months or for more than six months if an inoculum of 0.1 ml was used. This does not depend on a change in the age distribution, i.e. that the specimens stored for a longer time would have been from older patients because all the age groups were represented in this group in equal proportions.

If the inoculum was increased from 0.1 ml to 0.5 ml, however, the virus recoveries from some of the specimens stored for more than six months increased considerably. No attempts to increase the virus recoveries of freshly collected specimens by increasing the size of the inoculum have been made because during the latter part of 1955, at the time this method was introduced, there were very few cases of poliomyelitis.

To turn to the antibody studies, an increase in type 1 neutralizing antibodies was seen in 64 per cent of the paralytic cases and in 46 per cent of the non-paralytic ones.

Besides the increase in type 1 antibodies an increase was also

TABLE 5  
FREQUENCY OF INCREASE IN ANTIBODIES AGAINST TYPE 1 AND SIMULTANEOUSLY  
AGAINST TYPE 2 AND/OR TYPE 3

Type Of Illness	Number Of Paired Sera Tested	Antibody increase							
		Type 1		Type 1 And Type 2		Type 1 And Type 3		All Types	
		No	%	No	%	No	%	No	%
Paralytic poliomyelitis ..	85	54	64	17	20	18	21	7	8
Nonparalytic » ..	39	18	46	0	0	2	5	0	0

seen in type 2 and type 3 antibodies, especially in the paralytic group.

In one case there was an increase in type 2 antibodies only and in one case of 1956 in type 3 antibodies only, but in many cases there was a simultaneous increase in antibodies against two types and some times against all the three types (table 5).

From the patients showing an increase in either type 2 or type 3 antibodies only the corresponding virus was isolated. Type 1 virus was isolated from the majority of the cases, showing an increase in antibodies against type 1 and some or both of the two other types.

#### DISCUSSION

As was stated already in the introduction the virus recovery rate was low compared to corresponding figures obtained elsewhere (See 1), and the technique could be discussed at length. Attention may be called to some points, however. Thus higher virus recovery rates were seen among children according to previous concepts (4, 13). The prolongation of the time between the collection of specimens and the onset of symptoms seemed to reduce the recovery rate in accordance with a recent investigation by Svedmyr and his associates (13). It has to be remembered, however, that the number of specimens referred to here and collected late in the disease are very few, and thus the figures are not very reliable.

On the other hand the time of storage of the specimens before testing seemed to influence the outcome of the test considerably.

Thus specimens stored for more than six months gave as a rule, fewer positives than those tested immediately, independent by the age of the patient or the time of collection after the onset of symptoms.

It is therefore felt that prolonged storage of specimens may decrease the virus recovery rate, apparently due to the inactivation of virus. According to Kibrick, Enders and Robbins(4), however, several of their specimens tested were stored for more than six months, and virus was still recovered in high percentage. In several instances, however, the authors had used very large inocula corresponding to 10 to 60 times 0.1 ml, although it does not appear whether this large inocula were used predominantly for the specimens stored for a longer period. In the experiments presented here larger inocula were used only for these specimens and the recovery rate was considerably increased. It is probable therefore that besides several other factors one of the reasons for the diverging recovery rates so far obtained may be the difference in time of storage before testing.

An increase in antibodies was seen in 64 per cent of the paralytic and in 46 per cent of the non-paralytic cases. Several of the first blood specimens were collected fairly late in the disease and contained already antibodies in high titer, and thus an antibody increase could not be demonstrated.

This fact in addition with the low virus recovery rate thus resulted in that a virological diagnosis, based on either virus isolation or antibody increase, was established in only 79 per cent of the typical paralytic cases and in 50 per cent of the non-paralytic cases.

For this reason the number of non-paralytic cases caused by poliomyelitis virus in 1954 must actually have been higher.

Considering this the number of non-paralytic cases caused by the poliomyelitis virus in the non-epidemic year 1953 was high.

It is also evident that type 1 poliomyelitis virus dominates the picture both in paralytic and non-paralytic cases in this country at the present time.

During 1954 and especially 1955, however, a considerable number of cytopathogenic agents not typable by poliomyelitis immune sera have also been isolated both from paralytic and from non-paralytic cases. These agents have not been investigated

further, and it is therefore not known whether they belong to any of the known groups of cytopathogenic agents or not. Most of them were not cytopathogenic for HeLa cells, and the few which were did not behave like APC viruses. Attempts to propagate a few of the agents in suckling mice were unsuccessful.

The results of the antibody studies finally deserve some mention. In this study as well as in a previous one (10) a considerable number of sera showing an increase in neutralizing antibodies against type 1 virus also showed an increase in antibodies against type 2 and type 3 viruses. In many of these cases type 1 virus was isolated and in no case two or three antigenic types could be demonstrated. Thus the increase in antibodies other than type 1 seems to represent a heterotypic antibody response.

This question has lately attracted some attention (2, 8, 11, 12), and it has among other things been shown that several recently isolated poliomyelitis strains are ditypic or even tritypic (8). In this series an increase in type 3 antibodies appeared nearly as often as an increase in type 2 antibodies.

Studies in the immunity status of the population, for instance in this country, have shown a very similar incidence in antibodies against all the three types (6, 10) and this state of affairs may probably be partly explained by such a heterotypic antibody response.

#### SUMMARY

Some results obtained with the tissue culture technique in the diagnosis of poliomyelitis during the years 1953—1955 have been presented.

Among 136 strains of poliomyelitis viruses isolated 132 were of type 1, and four of type 2. One type 3 virus was isolated in 1956. In addition 46 strains of unidentified cytopathogenic agents were isolated.

It was shown that the virus recovery rate was higher in children than in adults. The recovery rate was lower if the specimens were stored long before testing than if they were tested immediately or within three months after collection. By enlarging the inoculum the recovery rate was considerably increased.

In several of the cases from which virus was isolated an in-

crease in homotypic antibodies was demonstrated. In a number of cases caused by type 1 virus, however, a heterotypic antibody response against both type 2 and type 3 viruses was seen.

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## CHOLINESTERASE IN DIPHYLLOBOTHRIUM LATUM AND TAENIA SAGINATA

by

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The most common source of cholinesterase (ChE) in the earlier investigations was blood serum, which was able to split butyrylcholine (BuCh) at the highest rate (18). The ChE activity of blood cell preparations (1) was associated with erythrocytes, since washed leukocytes were inactive against acetylcholine (ACh). In addition, erythrocyte ChE showed substrate specificity to certain choline acetylestere, thus differing distinctly from the behaviour of serum ChE.

Mendel and Rudney (11) concluded that there were two enzymes in the animal body capable of hydrolysing ACh: a »true» ChE, which acted only against the esters of choline, and a »pseudo» ChE which hydrolysed not only the esters of choline but a variety of non-choline esters as well. This classification of ChE:s corresponds approximately to the division into specific and non-specific types employed by certain investigators (12).

A decisive difference (11) between true and pseudo ChE was observed in the respect that true ChE displayed its maximum activity in low concentrations of ACh (less than 3 mg per 100 ml) and was inhibited in high concentrations, whereas pseudo ChE attained its maximum activity in high concentrations of ACh (above 300 mg per 100 ml). An eserine concentration sufficient to completely inhibit the hydrolysis of choline esters was even when thousandfold adequate to inhibit only in part the splitting of aliphatic esters (10).

Pioneers in research on ChE of invertebrates were Bacq and Oury (5), who were the first to demonstrate a strong ChE activity

in such worms as *Ascaris megalocephala*, *Distomum hepaticum*, and *Lumbricus terrestris*. In studying the ferment system of endoparasitic animals, there was found early (14) a significant ChE activity in the following parasites: *Planariae*, *Fasciola hepatica*, *Taenia pisiformis* and *Cysticercus pisiformis*. And it was also observed (9) that flatworms *Protyla* and *Planaria* were capable of significantly hydrolysing ACh even when decapitated. In his review Bacq (6) concluded that the motor nerves of the worms were in general cholinergic. It has been suggested (15) that in very many invertebrates, for instance in the annelids, ACh served as a neurohumoral transmitter of the nerve impulse into the muscle. In several cases eserine was found to potentiate the contraction caused by the stimulus. This is evidence for the necessity of ChE as an inactivator of the ACh liberated by nerve stimulation. Baldwin and Moyle (7) were unable to observe any sensitisation of muscle contraction of *Ascaris* to ACh by eserine treatment, and concluded that *Ascaris lumbricoides* do not contain ChE. This opinion was confirmed later (8), since it was found that the hydrolysis of ACh by *Ascaris lumbricoides* was negligible, and that *Schistosoma mansoni* and *Litomosoides carinii* were capable of hydrolysing ACh more definitely and a little BuCh. As ACh was split at a definitely higher rate than BuCh, it was concluded that the schistosoma and litomosoides homogenates contain acetylcholinesterase (AChE).

Since the fish tapeworm *Diphyllobothrium latum* (D.latum) is very common in Finland and the drugs used for its expulsion are very toxic, there is reason to study more closely the biological properties of this parasite and of *Taenia saginata* (T.saginata). It has been stated (13), that ACh caused relaxation in the cat tapeworm. On the other hand, the present author (16) found ACh in the intact tissues of *D. latum*. It therefore seems natural to investigate the possible presence of ChE in *D. latum* and *T. saginata*.

#### MATERIAL AND METHODS

Six *D. latum* and one *T. saginata* were used for the ChE determinations. Immediately after the parasites had been expelled they were washed and placed in a freezing box at  $-20^{\circ}\text{C}$ . They were usually stored in this condition for two weeks and then thawed out and minced in Ringer's solution prepared according to Augustinsson (4). A test tube homogeniser was usually employed for the mincing. For the preparation of a crude homo-



genate c. 1—2 g of fresh tissue and 2—6 ml of Ringer's solution were used. From each homogenate three determinations were made. In order to determine the non-enzymic hydrolysis one control test was made with each substrate. The carbon dioxide evolved by the worm homogenate itself was always measured before the experiments. The ChE activity was determined by the Warburg manometric method modified by Augustinsson (4). The method is based on manometric estimation of the volume of  $\text{CO}_2$  evolved from the bicarbonate-containing system by the acid formed in the ester hydrolysis. The volume of the reaction mixture has always been 2.00 ml; 1.60 ml of substrate solution was placed into the main compartment of the flask and 0.40 ml of enzyme solution into the side bulb. The ChE activity measured in an  $\text{N}_2\text{-CO}_2$  atmosphere at pH 7.4 is expressed in microlitres of  $\text{CO}_2$  per 30 minutes per mg of dry worm homogenate. This is the same unit as the  $b_{30}$  employed by Augustinsson and = total activity  $a_{30}$  minus non-enzymic hydrolysis.

## RESULTS

TABLE 1

ENZYMIC HYDROLYSIS OF ACh, MCh, BeCh, and BuCh BY DIPHYLLOBOTHRUM LATUM EXTRACTS.  $\mu\text{l CO}_2$  EVOLVED DURING 30 MIN. =  $b_{30}$ , CALCULATED FOR 10 MG DRY HOMOGENATE. SUBSTRATE CONCENTRATION: 0.25%

No. of Specimens	ACh		MCh		BeCh		BuCh	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
1	172	163	32	31	40	33	1	2
	157		26		35		5	
	160		34		23		0.2	
2	120	116	20	22	29	23	2	6
	112		23		24		1	
	116		24		17		16	
3	78	81	13	14	25	22	0	0
	81		12		21		0	
	83		17		21		0	
4	70	73	1	2	11	10	0	0
	75		1		16		0	
	74		3		4		0	
5	115	113	16	17	43	35	5	5
	110		16		30		5	
	114		18		32		4	
6	39	43	44	17	13	10	0	0.5
	50		4		7		1	
	41		4		10		0.5	
7	104	103	10	12	42	38	0.7	1.4
	102		13		38		1.2	
	104		14		35		2.4	

It will be seen from table 1 that the *D. latum* and *T. saginata* suspensions show definite activity against certain cholinesters. The activity is strongest against ACh, but acetyl- $\beta$ -methylcholine (MCh) also is hydrolysed but at a considerably lower rate. The hydrolysis of benzoylcholine (BeCh) takes place at a somewhat higher rate than that of MCh. BuCh is not hydrolysed at all or at a very low rate.

The activity-substrate concentration curves for the hydrolysis of ACh and triacetin (TA) by *D. latum* homogenates are shown in fig. 1. In graphs showing ChE activity the substrate concentration is usually expressed by the symbol pS ( $= -\log$ -molar concentration). The  $b_{30}$  on the ordinate is plotted against pS on the abscissa and each point is the mean of two determinations. The ChE of the crude homogenate displays its optimum activity against ACh at pS 2.0. The rate of TA hydrolysis, on the other hand, increases with increasing substrate concentrations.

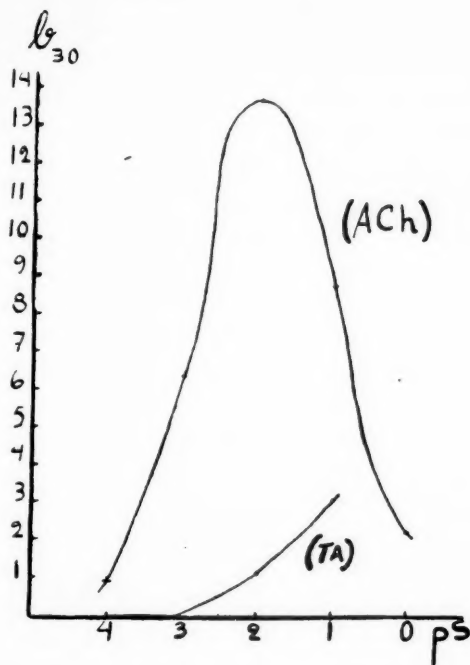


Fig. 1. — Activity=pS curves for the enzymic hydrolysis of ACh and TA by *Diphyllbothrium latum* homogenate.  $b_{30} = \mu\text{l CO}_2/30 \text{ min.}/\text{mg dry homogenate}$ .

## DISCUSSION

Of the substrates used in this investigation, ACh was the target of the strongest activity displayed by homogenates of *D. latum* and *T. saginata*. It may be regarded as probable that the enzyme in the worm homogenate which displays its activity against ACh is identical with that which splits MCh. According to the results obtained in this work, the rate of hydrolysis of MCh is about 2—31 per cent of the rate of ACh hydrolysis. Experience gained during the work, however, seems to indicate that had a young worm, or at least the youngest part of the parasite, always been available, the rate of hydrolysis of MCh would have been equivalent to 20—25 per cent of the ACh hydrolysis rate. Since the rate of MCh hydrolysis is generally known to be considerably lower than that of ACh, a strong ChE activity would be necessary; this is present in the younger tissues of the parasite. The results obtained supply evidence that the hydrolysis of BeCh takes place at a rate which is c. 10—38 per cent of the rate of ACh hydrolysis. An evaluation of the BeCh hydrolysis in the light of the ChE activity displayed by young and older tissues indicates that under optimum conditions BeCh is split at an average rate of 30—36 per cent of the rate of ACh hydrolysis. At the same time it appears very possible that the enzyme responsible for BeCh hydrolysis is totally different from that which splits ACh and MCh. In practice this may signify that *D. latum* and *T. saginata* contain at least two different enzymes, i.e., AChE and some enzyme similar to pseudo ChE (11) which is also termed non-specific ChE (12, 2, 3, 4). When we further consider that BuCh is split at a rate which is 0—6 per cent of the rate of ACh hydrolysis, the possibility cannot be wholly excluded that *D. latum* contains benzoylcholinesterase (BeChE), which was found in the liver of certain rodents (17). The use of inhibitors, which will not be discussed here, sheds further light in the above mentioned problem.

It is regarded that the activity-pS curve has a given shape according to the nature of the enzyme which hydrolyses ACh. Augustinsson, in his very detailed study (4), has described the relationship between substrate concentration and ChE activity by using various choline esters and ChE preparations from various sources. His results seem to provide conclusive evidence that the serum ChE,

which generally has been regarded as a non-specific enzyme, will give a nearly S-shaped activity-pS curve. Erythrocyte ChE, on the other hand, which represents the so-called specific type, has a bell-shaped (Haldane) activity-pS curve which differs from the curve exhibited by the serum ChE. This relationship is most evident when ACh is used as substrate, but MCh will also give with erythrocyte ChE a curve which rather is bell-shaped than S-shaped, although not as symmetrical as that obtained with ACh.

In studying the ChE activity of certain endoparasitic animals, Bueding (8) found AChE in *Schistosoma mansoni* and *Litomosoides carinii*. Using ACh and TA as substrates he also studied the effect of the substrate concentration on the activity displayed by worm homogenates. The hydrolysis of ACh, reaching its maximum at already a comparatively low concentration exhibited a humped activity-pS curve. In contrast, TA was hydrolysed by the schistosoma homogenate at an increasing rate parallel with the increase of substrate concentration. These observations and the low rate at which BuCh was split gave him reason to conclude that *Schistosoma mansoni* and *Litomosoides carinii* contain the so-called specific ChE. On the same grounds it may be regarded as probable that *D. latum* and *T. saginata* contain AChE, and that another, possibly non-specific ChE is responsible for the hydrolysis of BeCh. It is thought that the specific ChE of *D. latum* and *T. saginata* plays a role in the kinetics of the parasites.

#### SUMMARY

The writer has observed that the tapeworms *Diphyllbothrium latum* and *Taenia saginata* contain a specific acetylcholine-hydrolysing enzyme as well as another enzyme with ability to split benzoylcholine.

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## DETECTION OF A NEW ESCHERICHIA COLI TYPE, 55:B5:32 ASSOCIATED WITH INFANTILE DIARRHOEA

by

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Numerous reports have been published in recent years on the role of *Escherichia* bacilli in diarrhoeal diseases of infants. *E. coli* O groups 26, 55, 86, 111, 112a 112c, 124, 126, 127 and 128 have so far been found to be closely associated with infantile diarrhoea. Although O groups 25 (2, 11, 12), 119 (15) and 125 (3), have been isolated from cases of infantile diarrhoea, they have been observed only occasionally and additional investigations are necessary to evaluate the clinical importance of these groups. The K antigens of the *Escherichia* types associated with diarrhoea were found in all instances to be of the B variety. The antigens of the aforementioned *Escherichia* types are listed up to 1955 by Edwards and Ewing (4). In addition to non motile *E. coli* 55:B5 strains (5, 7, 15), motile strains with H antigens 2 (8), 4 (6), 6 (7), 7 (1), 8 (13), 10 (13), 11 (10) and 21 (10) have been demonstrated. After completing the investigation presented in this report, we noted that the type mentioned in the title has been reported by L. Le Minor et al. (1955). The three strains they described were originally isolated in Israel during 1953 and 1954.

The H antigens of *Escherichia* types associated with infantile diarrhoea are thus seen to vary greatly. The pathogenicity of the *Escherichia* types may not be associated with the H antigen, but the clarification of the proper antigenic formulae are of great value for the determination of the identity of strains isolated by

various investigators and also for the compilation of epidemiological data. It therefore seems motivated to report a new *E. coli* type 55:B5:32 isolated from an outbreak of diarrhoea in a nursery.

*Methods.* — The culture technique, the determination of the biochemical behavior of the isolated strains and the serological identification were performed in the same manner as described by one of us in an earlier report (6). Attempts were made to use sorbitol agar as originally suggested by Rappaport and Hening (14). The results were not encouraging and we did not therefore use sorbitol agar for the initial detection of *Escherichia* 55:B5 strains associated with the outbreak. The drug sensitivity tests were made using sensitivity tablets manufactured by Roskilde & Co. Denmark.

*Source of Materials.* — In April 1955 a diarrhoea outbreak occurred among 53 babies in a nursery caring for infants of tuberculous mothers in Tampere. Faeces specimens were taken from babies on 8 occasions during the period 25. 4.—2. 8. 1955. Thirty-three of the babies were less than 3 months old (14 less than 1 month), 14 were over three but less than 6 months and 6 were over 6 months old (none however, over 9 months). Increased stool frequency or abnormal stools were observed in 42 cases, 26 cases of which were positive for *E. coli* type 55:B5 while 16 were negative. The number of healthy babies was 11, from these this *E. coli* type was isolated in four cases. Apparently these four were healthy carriers. The isolated strains were found to possess H antigen 32 as will be presented below.

During January and February 1956, a control survey was carried out at the same nursery. At that time there were 46 babies in the nursery from whom specimens were taken on 3 occasions. Fourteen infants had diarrhoea or abnormal stools; six of them were found to be positive for *E. coli* 26:B6. Two healthy contacts were also found positive for the same type. None of the babies were found to harbor type 55:B5:32.

In all 59 strains of type 55:B5:32 were isolated during the first outbreak. An additional strain of the same type was isolated on January 16 th from an infant treated for diarrhoea at the Childrens Hospital of Lapland Province at Rovaniemi.

*Bacteriology and Biochemical Behavior.* — The 55:B5:32 colonies grown on different plates were not distinguishable from common *Escherichia* colonies.

All of the isolated strains promptly fermented glucose, lactose, maltose, arabinose, mannitol, adonitol, rhamnose and xylose and were late salicin positive. Sorbitol was attacked in 48 hours by all strains. With the exception of two strains which were found to be variable the strains also failed to ferment sucrose.<sup>1</sup> All strains pro-

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<sup>1</sup> All strains were dulcitol and inositol negative.



duced gas from glucose and mannitol and were indole positive, but none was able to liquefy gelatine. The strains did not utilise citrate or decompose urea. Voges-Proskauer tests were negative and methyl red positive for all strains. They also reduced potassium nitrate and were  $H_2S$ -negative. All strains were motile and with one exception did not haemolyse.

*Serological Properties.* — Attempts to determine the H antigens revealed that none of the 59 isolated strains possessed any of the known H antigens related to type 55:B5. The strains were therefore titrated with all the remaining known Escherichia H immune sera. The strains were found to give positive agglutination reactions with H 30 immune serum with titres 1/1600—1/3200 and with H 32 immune serum with titre 1/12,800, the titres of the two immune sera were 1/6,400 and 1/12,800 respectively. The H 30 and H 32 immune sera were therefore absorbed with H 30 and H 32 strains and titrated with strains Nos. 2740/55, 2751/55 and 29/56. After absorbing H 30 immune serum with strain 32, it was established that the agglutinins for H 30 remained (Table 1). After absorbing

TABLE 1

ABSORPTION AND AGGLUTINATION TESTS WITH H IMMUNE SERA OF ESCHERICHIA COLI H 30 AND H 32 TYPE STRAINS AND STRAINS NOS. 2740/55, 2751/55 AND 29/56

H Immune Sera	Absorbed with Living Culture	Titrated with Living Strain					
		H 30	H 32	2740	2751	29	H 1
H 30		6400	1600	3200	3200	3200	0
	H 30	0	0	0	0	0	0
	H 32	6400	0	0	0	0	0
H 32		100	25600	25600	25600	25600	0
	H 30	0	12800	12800	12800	12800	0
	H 32	0	0	0	0	0	0

*Escherichia coli* 6:K2a,2c:1 (Bi 7458/41) has been employed as negative control strain.

H 32 immune serum with strain H 30 the agglutinins for H 32 remained. Evidently the strains under test thus possessed the H antigen 32. As the *E. coli* 55:B5 subtype with H 32 antigen was a new type which had not been previously reported, cross-absorption tests were made to confirm the diagnosis of type 55:B5:32. For this purpose O, OB and H immune sera were prepared using two of

TABLE 2

ABSORPTION AND AGGLUTINATION TESTS WITH O AND OB IMMUNE SERA OF  
 ESCHERICHIA COLI 55:B 5:6 TYPE STRAIN 1064 AND STRAINS NOS. 2740/55  
 AND 2751/55

Immune Sera	Absorbed with Living or Steamed <sup>1</sup> Culture	Titrated with Strain							
		1064		2740		2751		Bi 7458/41	
		Living	Steamed <sup>1</sup>	Living	Steamed <sup>1</sup>	Living	Steamed <sup>1</sup>	Living	Steamed <sup>1</sup>
O 1064		20	1280	0	1280	0	1280	0	0
	1064	0	0	0	0	0	0	0	0
	2740	0	0	0	0	0	0	0	0
	2751	0	0	0	0	0	0	0	0
	Bi 7458/41	0	1280	0	1280	0	1280	0	0
O 2740		320	5120	320	5120	320	5120	0	0
	1064	0	0	0	0	0	0	0	0
	2740	0	0	0	0	0	0	0	0
	2751	0	0	0	0	0	0	0	0
	Bi 7458/41	160	2560	160	5120	160	2560	0	0
O 2751		320	2560	320	2560	160	2560	0	0
	1064	0	0	0	0	0	0	0	0
	2740	0	0	0	0	0	0	0	0
	2751	0	0	0	0	0	0	0	0
	Bi 7458/41	160	2560	160	2560	160	5120	0	0
OB 1064		640	5120	1280	2560	1280	5120	0	0
	1064	0	0	0	0	0	0	0	0
	2740	0	0	0	0	0	0	0	0
	2751	0	0	0	0	0	0	0	0
	Bi 7458/41	320	2560	320	2560	640	5120	0	0
OB 2740		160	2560	160	2560	160	2560	0	0
	1064	0	0	0	0	0	0	0	0
	2740	0	0	0	0	0	0	0	0
	2751	0	0	0	0	0	0	0	0
	Bi 7458/41	160	2560	160	2560	80	1280	0	0
OB 2751		640	40	640	40	1280	40	0	0
	1064	0	0	0	0	0	0	0	0
	2740	0	0	0	0	0	0	0	0
	2751	0	0	0	0	0	0	0	0
	Bi 7458/41	1280	80	1280	160	1280	40	0	0

<sup>1</sup> kept 2 ½ hours at 100° C.

*Escherichia coli* 6: K2a,2c:1 (Bi 7458/41) has been employed as negative control strain.

TABLE 3

ABSORPTION AND AGGLUTINATION TESTS WITH H IMMUNE SERA OF ESCHERICHIA COLI H 32 TYPE STRAIN AND STRAINS NOS. 2740/55 AND 2751/55

H Immune Sera	Absorbed with Living Culture	Titrated with Living Strain			
		H 32	2740	2751	Bi 7458/41
H 32		12800	12800	12800	0
	H 32	0	0	0	0
	2740	0	0	0	0
	2751	0	0	0	0
	Bi 7458/41	12800	12800	12800	0
2740		3200	3200	3200	0
	H 32	0	0	0	0
	2740	0	0	0	0
	2751	0	0	0	0
	Bi 7458/41	3200	1600	1600	0
2751		12800	6400	12800	0
	H 32	0	0	0	0
	2740	0	0	0	0
	2751	0	0	0	0
	Bi 7458/41	6400	6400	6400	0

*Escherichia coli* 6:K2a,2c:1 (Bi 7458/41) has been employed as negative control strain.

the isolated strains 2740/55 and 2751/55. O and B cross-absorption tests were performed with the *E. coli* 55:B5:6 type strain 1064 and the two of the isolated strains, and their immune sera. H absorption tests were made using the strain H 32. Tables 2 and 3 show that sera absorbed with suspensions of living cultures and cultures steamed at 100° for 2½ hours gave similar titres when titrated with these strains. In cross-absorption tests, the strains were able to exhaust quantitatively all agglutinins from each other's immune sera.

**Drug Sensitivity.** — On primary isolation all the 60 *E. coli* 55:B5:32 strains were found to be resistant to sulphathiazole, but sensitive to streptomycin and chloramphenicol. Three of the isolated strains were moderately sensitive to oxytetracycline. An increase in resistance against oxytetracycline was observed during the treatment in 5 cases.

## SUMMARY

A new serotype of *Escherichia coli* is described for which the antigenic formula was found to be 55:B5:32. The appearance of this serotype coincided with an outbreak involving 42 cases of infantile diarrhoea in a nursery at Tampere. Twenty-six diarrhoeal infants and four healthy carriers were found to be positive for this type. The same serotype was also isolated from an infant treated for diarrhoea at the Lapland Childrens Hospital.

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## SERUM PROTEINS IN DEHYDRATION IN YOUNG RATS

by

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(Received for publication June 5, 1956)

The plasma proteins play a great part in the regulation of the water balance in the animal organism. It may logically be assumed that dehydration may induce changes in the colloid chemistry of the blood. Some investigations on this subject have been performed on adult individuals. As far as we are aware, the only studies concerning childhood deal with the plasma proteins in diarrhea, pylorospasm or other pathologic conditions of that kind. Our object has been to investigate whether any quantitative changes occur in the albumin-globulin ratio and the total serum proteins of young rats deprived of food and drink, and then to study the effects, if any, of giving either food or drink alone.

### METHODS

As experimental animals, young albino rats of the Sprague-Dawley strain, 12—15 days old, were used. At this age they had already got their coat of hair, and the eyes had just opened. The animals were kept in a thermostat at 33—34°C. Water was administered 3—4 times a day in doses of 0.5 ml with a polyvinyl tube passed into the stomach. In the same way, the animals getting food were fed three times a day with 0.3—0.4 ml of an emulsion of edible oil in 2 per cent saline solution saturated with regard to glucose and peptone, its caloric value being about 4 cal/ml. The control animals were killed 4—6 hours after they had been taken from the dam. The whole group without food and drink and most of those getting water fasted 3 days. A few were kept in the thermostat for 6 days in order to follow the further development of the situation. Of the first litter of rats getting food alone, one half died in the first two days and the rest were in

very poor condition. Consequently, further experiments of this kind were abandoned.

The blood was collected after decapitation. An average of 0.3—0.4 ml per animal was obtained. One part of the analyses was made with an Antweiler microelectrophoresis apparatus. The serum was in the dialysis chamber 1—2 hours. It was then diluted to 1: 1.5 with the buffer solution. In the run 1.8 mA current was employed for 15 minutes (U 50—70 V). Most of the analyses were made with paper electrophoresis. 0.020—0.030 ml of serum was pipetted on a Munkell 20/150 paper strip 4 cm wide. The voltage was about 10 V/cm and the run lasted 4 hours. The staining was performed with bromphenol blue, following the procedure of Durrum (Durrum et al. 1955). After elution the fractions were measured quantitatively by means of a Unicam spectrophotometer. Since the number of analyses made with the Antweiler microelectrophoresis apparatus was not sufficient for calculating conversion factors to characterize the different coloration of albumins and globulins, relative values taken straight from the extinctions were used. From every experimental animal 3—6 paper electrophoreses were made, whereas in the Antweiler it was usually necessary to pool the blood of two animals for a single analysis.

All the time barbital buffer of pH 8.6 was used, with molarity 0.12 in free electrophoresis and 0.06 in paper electrophoresis. The total protein was determined by the biuret method as modified by Weichselbaum (cited by Levén 1952). The extinction curve was constructed from values for normal human serum, the total protein of which was determined by the micro-Kjeldahl method.

## RESULTS

With the Antweiler microelectrophoresis apparatus the following data were obtained:

TABLE 1  
THE PERCENTAGE OF ALBUMINS AND GLOBULINS

Litter	Controls		Without Food		Without Water	
	Alb.	Glob.	Alb.	Glob.	Alb.	Glob.
1	69.0	31.0				
	68.0	32.0				
	69.0	31.0				
	77.0	23.0	80.0	20.0	78.0	22.0
2—3	71.0	29.0			65.5	34.5
	76.0	24.0			65.5	34.5
4			73.5	26.5		
			78.5	21.5		
			76.5	23.5		
Mean	71.6	28.4	77.2	22.8	69.7	30.3

TABLE 2

THE PERCENTAGE OF ALBUMINS AND GLOBULINS AND STANDARD ERROR

Litter	Age Days	Controls			Fast with Water			Fast without Water		
		Alb.	Glob.	Se.	Alb.	Glob.	Se.	Alb.	Glob.	Se.
1	14	53.7	46.3	1.0	53.8	46.2	0.7	61.9	38.1	2.7
					59.0	41.0	1.7			
					57.8	42.2	1.3			
					58.6	41.4	1.4			
3	14	53.2	46.8	1.0	66.1	32.9	0.6	60.5	39.5	2.1
		52.2	47.8	0.7	59.6	40.4	0.9	59.7	40.3	1.1
4	13	59.0	41.0	3.0	63.0	37.0	1.0	54.3	45.7	1.0
		55.5	44.5	1.2	52.0	48.0	1.2			
5	12	50.7	49.3	1.9	61.4	38.6	0.4	53.7	46.3	1.0
		50.2	49.8	1.0	55.1	44.9	1.3	53.8	46.2	1.2
6	12	51.1	48.9	0.2				48.2	51.8	1.4
		52.7	47.3	1.4						
7	15	56.0	44.0	0.7				52.0	48.0	1.2
		57.4	42.6	0.9						
Mean		53.8	46.2		58.7	41.3		55.6	44.0	
sd.		2.7			4.5			4.4		

6 DAYS WITH WATER			
	Alb.	Glob.	Se.
rat 1	57.0	43.0	0.6
rat 2	48.6	51.4	0.9
rat 3	59.9	40.1	1.2
Mean	55.2	44.8	

$$\bar{x} = \frac{\sum x_v}{n} \quad se. = \frac{\sum x - x_v}{n} \quad sd. = \sqrt{\frac{\sum (x - x_v)^2}{n-1}}$$

TABLE 3

Controls	3.8	4.0	3.8	4.1	Mean 3.9 g/100 ml
Fast with Water	4.6	4.8	5.1		Mean 4.8 g/100 ml
Fast without Water	6.6	5.7	6.6	7.8	Mean 6.7 g/100 ml

In our experiments, the heading albumin includes the  $\alpha_1$ -globulin. It was remarkable that with the rat serum, in contrast to human serum, the different globulin fractions did not separate satisfactorily from one another.

In the paper electrophoresis, on the other hand, far better success was obtained with rat serum. Yet it was limited to determining the



total globulin only. By qualitative examination one could discern, after the  $\alpha_2$ -globulin, a distinct and large  $\beta$ -globulin fraction followed by far smaller, less distinct  $\gamma$ -globulin. The results are presented in table 2.

The total protein values are collected in table 3.

Some animals in every group were autopsied, but no signs of oedema were observed.

#### DISCUSSION

On the basis of the foregoing, the following conclusions may be drawn:

Food without water proves lethal in a far shorter time than fasting without food and drink. One reason for this in the experiments here described was perhaps the high saline concentration of the nutrient solution, in which the inorganic ions necessary for a rat were present in amounts adequate to its caloric value. Similar investigations have previously been done with adult rats (Dicker 1949) and cows (Wehmeyer 1954) in order to examine, among other things, possible changes in the blood proteins under these conditions. Then, however, forced feeding was not employed and as a consequence, the animals soon refused to eat anything. In the former investigation a 10 per cent increase in the total protein was observed after 6 days, in the latter a corresponding observation was made after 2 days. The increase was largely confined to the globulin fraction.

Three days' fasting with water caused a relative decrease of 10 per cent in the globulin fraction. There was no further change during the subsequent three days. For comparison's sake it may be stated that in corresponding situation in adult rats the albumin-globulin ratio remained unchanged during the first three days, then increasing to the of the week from 2.0 to 2.4 (Kohn et al. 1950). With human volunteers results pointing more clearly in the same direction have been achieved. Sundermann has reported on a 45 days' fast (Sundermann 1947). At the end there were 5.0 g albumins and 1.2 g globulins per 100 ml in the plasma. In contrast, some other authors have observed only slight changes, albeit during a far shorter time (Taylor et al. 1950).

Some differences were observable between the reactions of the

various animals, as in apparent from the increased standard deviation values in the fasting groups.

The total protein increased by more than 50 per cent in the group without food and drink. This markedly exceeds the value given by Dicker. In severe infantile gastroenteritis total protein values of more than 8 per cent have been observed, whilst the normal average at this age is 4.7 per cent (Hallman et al. 1952). When fasting, an adult rat drinks as much as 3—5 per cent of its body weight of water daily (Dicker 1949). Although the amounts given by us were nearly twofold, signs of a slight haemoconcentration could be noted. Perhaps, the amount of water was insufficient, but, on the other hand, the phenomenon has previously been attributed to the mild acidosis due to starvation (Taylor et al. 1949). A certain amount of caution is perhaps called for regarding the total protein values obtained by us, owing to the small number of analyses and to the fact that the 0.2 ml of serum necessary for the biuret reaction was not always available, measuring errors hence playing a greater part.

A sharp distinction has to be made between a complete fast and the various stages of malnutrition. To these the organism reacts in an entirely different way (Gülzow 1947).

When staining the serum proteins of young rats in paper electrophoresis with bromphenol blue according to Durrum's method, the conversion factor 1.3 for globulins could not be used to obtain agreement with the results obtained with free electrophoresis. Rather, the globulins seemed to color better, as is seen for instance from the control runs: In Antweiler the mean values of the globulins were 28.4 per cent and in the paper electrophoresis 46.2 per cent without any conversion.

#### SUMMARY

In order to gain further insight into the problem of dehydration and starvation in infancy, two week old rats were kept for three days without food and their serum proteins were then analyzed with the Antweiler microelectrophoresis apparatus, by paper electrophoresis, and by the biuret method. In rats getting no drink either, a marked haemoconcentration could be observed but no changes in the albumin-globulin ratio. In those getting water the

relative amount of the globulins decreased by 10 per cent. Food without water proved fatal in a short time. The coloration of the proteins with bromphenol blue differed from that of the proteins of human serum.

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## SUCCINIC DEHYDROGENASE ACTIVITY IN MALE ACCESSORY SEX ORGANS OF INTACT, TESTOSTERONE- TREATED AND CASTRATED RAT

A HISTOCHEMICAL STUDY

by

MATTI TUOVINEN and JUHANI RAPOLA

(Received for publication June 5, 1956)

Succinic dehydrogenase activity of the male accessory sex organs has escaped any notice in previously published studies concerned with the histochemical distribution of this enzyme in animals (2, 3, 6). In connection with our earlier study we found that the administration of testosterone propionate seemed to have a stimulating effect on the histochemically demonstrable succinic dehydrogenase activity of the seminal vesicle of rat (8).

The study now reported was carried out in order to investigate the succinic dehydrogenase activity in the prostate, coagulating gland and seminal vesicle of rat, as well as the effect of testosterone treatment and castration on the enzymatic activity in these organs.

### MATERIAL AND METHODS

Twenty male albino rats of the Wistar strain, weighing about 230 g, were used. Ten of them served as intact controls. Five rats were castrated under a slight aether anaesthesia 30 days before sacrifice. Five rats received daily 1 mg of testosterone propionate (Organon) intramuscularly over a period of 15 days. The animals were sacrificed by decapitation and then bled.

For the demonstration of the succinic dehydrogenase activity the histochemical method of Seligman and Rutenburg (7) was followed using neotetrazolium as an indicator for succinic dehydrogenase. The prostate, coagulating glands and seminal vesicles were immediately removed, frozen and sectioned with a freezing microtome at  $40\ \mu$  simultaneously with a control specimen, i.e. a castrated or testosterone-treated animal together with the corresponding control. The frozen sections were dipped into incubation vials kept at a constant temperature of  $38^{\circ}\text{C}$ . The incubation time was 30 minutes.

## RESULTS

The mean weights of the animals and the absolute and relative weights of the organs are given in Table 1.

TABLE 1

	Controls	Testosterone Group	Castrated
Body weight (g) .....	$233 \pm 15$	$231 \pm 10$	$222 \pm 16$
Weight of prostate (mg) .....	$512 \pm 13$	$665 \pm 20$	$88 \pm 6$
Weight of prostate (mg/g of body weight) .....	$2.17 \pm 0.2$	$2.87 \pm 0.2$	$0.39 \pm 0.1$
Weight of coagulating glands (mg)	$103 \pm 5$	$183 \pm 7$	—
Weight of coagulating glands (mg/g of body weight) .....	$0.44 \pm 0.2$	$0.81 \pm 0.2$	—
Weight of seminal vesicles (mg) ..	$483 \pm 20$	$1310 \pm 112$	$76 \pm 6$
Weight of seminal vesicles (mg/g of body weight) .....	$2.07 \pm 0.2$	$5.89 \pm 0.6$	$0.34 \pm 0.1$

(The coagulating glands of the castrated group are included in the weights of the seminal vesicles because of the minute size of the glands.)

The absolute and relative weights of the prostate, coagulating glands and seminal vesicles of the castrated and testosterone-treated animals differed significantly from the values of the controls.

In the histological demonstration of succinic dehydrogenase activity the following observations were made:

*The Prostate.* — A purple staining was seen in the sections from the controls. In some places a strong activity was observed in ducts

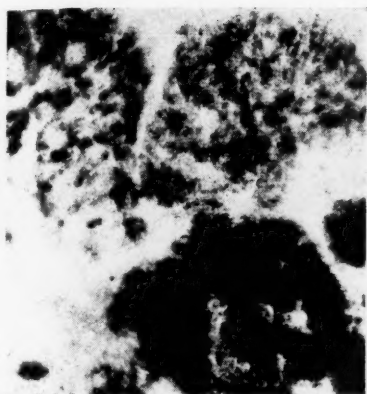


Fig. 1. — Prostate of a testosterone-treated rat. (110  $\times$ )

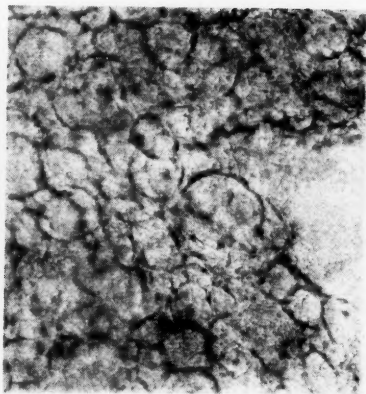


Fig. 2. — Prostate of a castrated rat. (110  $\times$ )



Fig. 3. — Seminal vesicle of a testosterone-treated rat. (110  $\times$ )

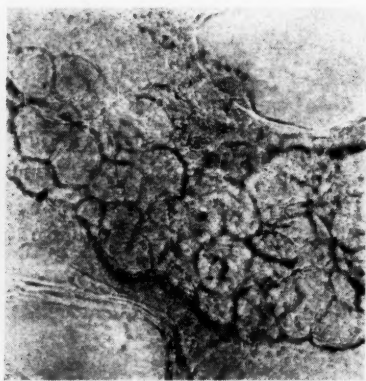


Fig. 4. — Seminal vesicle of a castrated rat. (110  $\times$ )

and in the epithelium lining the glands. No difference was found between the ventral and dorsal lobes of the organ.

In the testosterone-treated group an intense granulation was seen in the epithelium of the glandular structure and of the ducts. In the tissue between the glandular portions patches of smooth muscle were also stained (Fig. 1.). No difference was found between the ventral and dorsal lobes of the organ.

The sections of the castrated rats remained generally unstained, only in small areas a slight purple hue was seen (Fig. 2.).

*The coagulating gland.* — The slides from the control animals showed a clear activity in the epithelial cells. The activity was distinctly increased in the testosterone-treated group.

*The seminal vesicle.* — The epithelial cells showed a marked activity in the control animals. The treatment with testosterone seemed to increase the enzymatic activity. The cytoplasm of the epithelial cells showed a deposit of bluish purple granules of formazan. The colour of the sections in their entirety including muscle and connective tissue fibres was more intense than in control specimens. The changes induced by testosterone were more remarkable in this organ than in the other two glands (Fig. 3.). The sections from the castrated animals remained unstained (Fig. 4.).

#### DISCUSSION

According to our results the male accessory sex organs have a distinct succinic dehydrogenase activity. Under our experimental conditions the testosterone propionate treatment seemed to increase the histochemically demonstrable enzymatic activity in these organs. The castration resulted in a disappearance of the succinic dehydrogenase activity. Our observations on the decrease of enzymatic activity in male accessory sex organs after castration concur with the earlier findings obtained with biochemical methods (1, 5). The biochemical results of previous investigators also suggest that the utilization of carbohydrates via the citric acid cycle of Krebs, in which succinic dehydrogenase is an essential link, may be stimulated in male accessory sex organs by the influence of androgenic hormones (4), a finding which is also in agreement with our present histochemical observations.

#### SUMMARY

Histochemically demonstrable succinic dehydrogenase activity of the prostate, coagulating gland and seminal vesicle of intact, castrated and testosterone-treated rats was investigated. A clear succinic dehydrogenase activity was observed in the organs of intact control animals. The testosterone treatment resulted in a distinct increase in the activity. Only a slight or no activity was found in the accessory sex organs of the castrated animals.



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## OBSERVATIONS ON THE USE OF RADIOACTIVE GOLD (AU 198) IN THE TREATMENT OF MALIGNANT TUMOURS OF THE LUNGS AND PLEURA<sup>1</sup>

by

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(Received for publication June 14, 1956)

In patients arriving for treatment for malignant tumours of the lung the disease frequently is so far advanced that removal of the tumour cannot be considered. Furthermore the patient's poor physical condition and cardiopulmonary insufficiency may also prevent operation. Primary contraindications for surgery are thus present in a considerable proportion of the cases. In many patients the inoperability of the tumour becomes evident only on the operating table.

For the treatment of inoperable malignant tumours of the lung and carcinosis of the pleura, certain therapeutic centres have employed radioactive colloidal gold preparation Au 198, the radiation properties of which have been considered to be suitable for this purpose.

The gold preparation may be used for infiltration of the primary tumour or of lymph node metastases during surgical operation. In cases of pleural carcinosis it may be injected into the pleural cavity. Its direct application into the tumour tissue is also possible

<sup>1</sup> We wish to express our gratitude to Professor Sakari Mustakallio, M.D., Head of the Department of Radiation Therapy of the Helsinki University, for giving us the benefit of his great experience in our planning and carrying out the present work. We also appreciate the opportunity we have had to consult Phil. Mag. K. E. Salimäki, physicist at the Department of Radiation Therapy, concerning dosages and radiation measurements.

by bronchoscopy. If the size of the particles is increased, the radioactive substance may also be injected into the pulmonary artery in the involved area, the substance then being retained in the capillary network of the area. When placed in a rubber bag it may be held for a specified time adjacent to the diseased tissue.

Reports on the use of radioactive gold preparation have been published in the past few years in both Anglo-American and continental literature. In addition to other radioactive substances Allen, Hempelman and Womack (1945) employed it in colloidal form in local infiltration therapy. In 1947 Hahn and his collaborators described the use of Au 198 for the infiltration of tumours. Müller was the first to use, in 1949, intrapleural isotope therapy. He observed that the gold preparation greatly diminished the formation of exudate in cases of pleural carcinosis. Kniseley and Andrews (1953) also considered the preparation suitable for the management of fluid effusion in pleural and peritoneal carcinosis (cf. also Müller, 1955). Their series comprised 30 patients and the maximum single dose was 187 mc. At postmortem examination they observed fibrosis, petechiae and tissue necroses on the serous membranes. Andrews, Root and Kniseley (1953) found only minor amounts of isotope in the urine and the liver, whereas large amounts were present in the lymph nodes in adjacent regions. They did not observe a selective effect on tumour tissue. In their opinion the decreased exudation after application of the preparation was a result of fibrosis of the tumour surface, reinstated patency of the lymph channels and shrinkage of the cavity.

A number of other investigators (Schick and Bloor, 1954; Kent, Moses, Ford, Kutz and George, 1954; Wheeler, Jaques and Botsford, 1955) treated pleural carcinosis with injections of Au 198 and similarly observed a diminished exudation.

Possible unfavourable effects arising from the use of radioactive gold were studied by Andrews, Root, Kerman and Bigelow (1953). Among such effects nausea, vomiting and lassitude usually were not marked and lasted for a few days only. Inhibition of haematopoiesis was observed but it was mild and transitory. Some amount of the isotope which had entered the blood circulation was stored in the liver or the spleen or was excreted in the urine. However, the amount was too insignificant to produce any damage.

Since in animal experiments carried out by us the radioactive

gold preparation was not found to injure normal tissues we began to use gold therapy in inoperable cases of tumours of the lungs and pleura.

#### WRITERS' SERIES

*Methods and Material.* — An apparatus for the application of the radioactive gold preparation was designed with the purpose of protecting the operators from unnecessary exposure to radiation. A simple construction and easy transportability were guiding principles. The construction of the apparatus will be seen in Figs. 1 and 2.

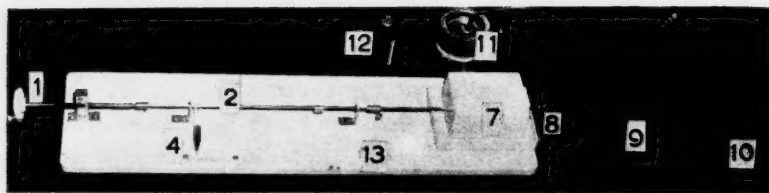


Fig. 1. — Apparatus used for the application of Au 198. Parts numbered as follows: 1. Rotating knob of piston rod. 2. Piston rod. 3. Injection syringe, 20 cc. 4. Metal pointer and scale. 5. Hinged support for piston rod, with screw threads. 6. Case of plexiglass. 7. Lead chamber for injection syringe. 8. Three-way stopcock. 9. Fine-calibre plastic tube. 10. Injection needle. 11. Injection bottle for Au 198 preparation, in lead container. (Shown with cover of container removed.) 12. Injection bottle containing physiological saline. 13. Stand on which apparatus is mounted.

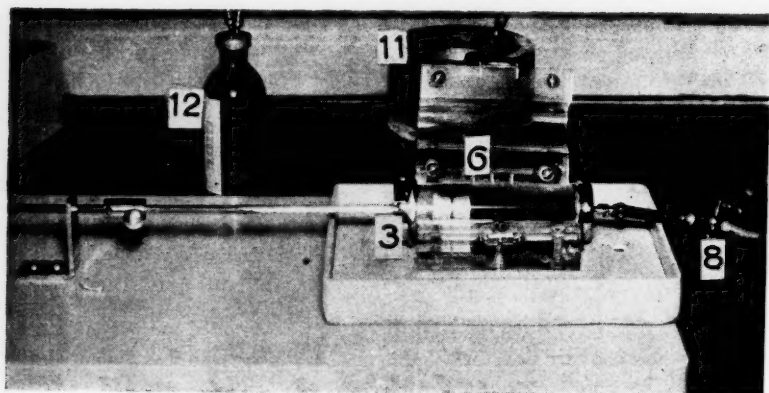


Fig. 2. — Apparatus with lead chamber removed and plexiglass case open. Numbers as in fig. 1.

The technique of injection was as follows: A 20 cc Record injection syringe was attached securely to a stand by means of a transparent plexiglass case and was protected by a heavy lead chamber. With the aid of a long piston rod the gold solution in the syringe was injected through a fine-calibre plastic tube into the target. A metal pointer mounted on the piston rod indicated on a scale the amount of solution remaining in the syringe as the injection proceeded. By means of a three-way stopcock on the plastic tube it was possible to refill the injection syringe with colloidal gold preparation or to dilute the contents with physiological saline. The connecting tubes were as fine as possible (inner diameter 2 mm) in order to minimize the amount of radioactive solution in the tubes since these attachments could not be placed within the lead cover during the administration of the injection. The connecting tubes had a volume of 2 cc. On performance of the injection one of the operators held the injection needle, mounted at the end of the plastic tube, by means of long pincers, while an assistant attended to the apparatus.

The injection bottle containing the gold solution was placed in a heavy lead container, which is shown with the cover removed in Figs. 1 and 2. The piston rod, which was furnished with screw threads, could be released from the hinged support (shown on the left side of the stand in Fig. 1) and the syringe could be filled or emptied by drawing out or pushing in the piston. When the hinged support was closed the filling and emptying of the syringe took place by turning the rotating knob at the end of the piston rod.

All the joints in the apparatus were carefully tightened before the injection was started, to prevent leakage during the application.

Injections of radioactive colloidal gold solution were given with this apparatus to eleven patients. The amount of gold preparation injected at a time ranged from 80 mc to 120 mc. In nine cases the injection was made in connection with thoracotomy. In two of these cases a palliative pneumectomy was performed and the colloidal gold preparation was injected into metastases in the mediastinum at the completion of the surgical procedure. In the case of one patient, from whom large amounts of soft tumour mass was removed because of pleural sarcomatosis, the Au 198 solution was injected into the walls of the pleural cavity. At thoracotomy the pulmonary neoplasms of six patients were

No.	Age Years	Sex	Finding at Operation	Type of Tumour	Surgical Procedure	Amount of Au 198	Mode of Injection	Follow-Up
1	53	♀	Fist-sized tumour extensively infiltrating the mediastinum	Undiff. ca	Thoracotomy explorativa	100 mc	Into tumour tissue and pleural cavity	Died 11 months after injection
2	31	♂	Several large necrotic masses in pleural cavity	Fibro-sarcoma	Evakuatio partialis tumoris	100 mc	Into walls of pleural cavity	Died 8 months after injection
3	69	♂	Tumour; pleural carcinosis over wide area	Adenoca	Thoracotomy explorativa	100 mc	Into tumour tissue and pleural cavity	Died 6 days after injection (embolism)
4	56	♂	Large tumour in lower lobe; metastatisation in mediastinal lymph nodes	Squamous cell ca	Pneumektomia palliativa	100 mc	Into mediastinal lymph nodes	Died 10 months after injection
5	50	♂	Pleural carcinosis following pneumectomy	Squamous cell ca	Punctio pleurae	1) 80 mc 2) 120 mc	Intrapleural injection made twice	Died 10 months after injection
6	52	♂	Fist-sized tumour with infiltration of mediastinum	Adenoca	Thoracotomy explorativa	120 mc	Into tumour tissue	Died 6 months after injection
7	48	♂	Small tumour in middle lobe; large number of enlarged lymph nodes in mediastinum	Squamous cell ca	Thoracotomy explorativa	100 mc	Into tumour and metastases	Alive 11 months after injection; process persists
8	56	♂	Fist-sized tumour in lung; large number of enlarged lymph nodes in mediastinum	Adenoca	Thoracotomy explorativa	100 mc	Into tumour and metastases	Died 8 months after injection
9	64	♂	Fist-sized tumour; large masses of lymph nodes in mediastinum	Squamous cell ca	Thoracotomy explorativa	100 mc	Into tumour and lymph nodes	Alive 12 months after injection; process persists
10	36	♂	Plum-sized tumour in hilar region; metastases in mediastinal lymph nodes	Undiff. ca	Pneumektomia palliativa	100 mc	Into hilar region of mediastinum	Died 7 months after injection
11	53	♂	Pleural carcinosis	—	Punctio pleurae	120 mc	Into pleural cavity	Died 5 months after injection

found to be inoperable and injections of Au 198 were made both into the primary tumour and the metastases that were found. One patient had undergone pneumectomy six months previously for carcinoma of the lung. He was re-admitted because of pleural carcinosis and received injections of gold solution into the pleural cavity in connection with aspirations made at intervals of two weeks. The last patient had pleural carcinosis but had not been operated upon and received gold injection therapy in connection with pleural aspiration.

After the injection the migration of Au 198 from the area of application was followed with a Geiger counter. Especial attention was paid to the readings from the liver and spleen areas and from the urine.

One year after treatment, follow-up data have been obtained for all the patients who received this treatment (cf. table 1).

*Results.* — Data on the series of cases is given in table 1, which shows the age and sex of the patients, findings at operation, type of tumour, procedure performed, amount of gold preparation injected, mode of injection, and follow-up data.

In following with the Geiger counter the localisation of the radioactivity on the first few days after the injection it was observed to remain within the injection area. Au 198 injected into the pleural cavity was found to be localised in the basal parts of the pleural cavity. No accumulation of gold in the liver, spleen or kidneys was observed. In two patients small but clearly demonstrable amounts were present in the urine; these were patients who were given injections into the mediastinal lymph nodes. The preparation in the doses used appeared to have no effect on the blood picture. The intensity of radiation declined rapidly, in conformity with the statements in literature, and it disappeared practically completely after ten days.

Observation of the clinical recovery of the patients revealed no complications or unfavourable effects ascribable to this treatment. Within three weeks the patients were in a satisfactory condition to leave the hospital and to be transferred to the Radiation Therapy Clinic for roentgen radiation. A male patient aged 69 died with acute symptoms of embolism six days after thoracotomy and Au 198 application. Further details on the cases will be seen in table 1.

It is very difficult to evaluate, especially in a small series of



cases such as the present, the benefit derived from the application of Au 198. The prognosis in these cases was grave in any case, but it is possible that application of radioactive gold had a certain palliative effect, objective evaluation of which naturally is difficult. Approximately one year after the gold injection procedure, nine patients in our series are dead and two alive. Two of the patients who received the preparation for pleural carcinosis have, at least, had the benefit that exudation decreased and the intervals between aspirations could be extended.

#### DISCUSSION

In the treatment of patients with malignant tumours of the lungs the possibility of surgical treatment is limited. Furthermore, even what may seem a radical removal of the growth may frequently be followed by local regrowth or distant metastases. After the failure of radical surgical treatment, radiation therapy must be resorted to for destruction of the primary tumour or the metastases. By using radioactive isotopes it is possible to control and localise the effect of radiation in a manner different from that possible with roentgen and radium therapy. For example, in connection with explorative thoracotomy it is possible to use isotopes to obtain a radiation effect which may be important for the prevention of metastases. Treatment may later be supplemented with roentgen therapy. Of the radioactive preparations, colloidal gold Au 198 is so far the most serviceable for the treatment of malignant tumours.

For this therapy we selected patients with malignant tumours of the lungs for whom radical resection or removal of the growth was no longer available. The dosage of radioactive gold varied from 80 mc to 120 mc. The injection was made directly into the tumour tissue and the mediastinal lymph nodes or into the pleural cavity. Geiger readings made after the injection showed that the injected gold remained at the site of injection, with the exception that gold applied into the pleural cavity moved by the law of gravity to the basal parts of the cavity. For this reason it is recommended that patients with pleural carcinosis should be kept recumbent in different positions in order to ensure a more even distribution of the preparation to the various parts of the

pleural cavity. In two cases, in which the injection was made into the mediastinal nodes, minor amounts of gold were demonstrated in the urine. No radiation, on the contrary, was noted in the region of the liver, spleen or kidneys or in the peripheral parts of the body. According to Andrews, Root, Kerman and Bigelow (1953), some small inhibition of haematopoiesis has been observed following the application of gold preparation. We found no changes of this kind in our series, nor were there any pathological signs in the liver function tests.

It is difficult to draw conclusions concerning the advantages of radioactive gold preparation since no reliable criteria are available to us. Of the eleven patients with malignant disease of the lung or pleura, nine died within one year from the procedure. Following the gold therapy the patients were transferred for roentgen therapy, and this also makes analysis of the effects of Au 198 difficult. Our series of cases, furthermore, was too small to permit us to draw any conclusions. However, it is known that in two cases in which frequent aspirations of the pleural cavity were necessary because of pleural carcinosis, there was a definite prolongation of the intervals between aspirations after gold therapy. Observations of a similar kind have been reported in the literature (Andrews, Root and Knisely, 1953; Schick and Bloor, 1954; Kent, Moses, Ford, Kutz and George, 1954; Wheeler, Jaques and Botsford, 1955). Such alleviations in the condition of a patient with malignant disease are, even if seemingly insignificant, indeed welcome.

On the basis of reports in the literature and of our own series it appears that the application of radioactive substances for therapeutic purposes must be regarded as insufficient for the destruction of carcinoma with the methods available at present (Lamerton *et al.*, 1955). It is difficult to reach an adequate concentration. Radioactive gold preparation may possibly prove beneficial as an adjunct to surgical treatment, especially for the prevention of distant metastases. When used in conjunction with explorative thoracotomy in inoperable cases, radiotherapy can thus be started immediately, instead of at least two weeks after the operation. As a palliative measure, the preparation is beneficial by decreasing the exudation in cases of pleural carcinosis.

## SUMMARY

The writers have employed radioactive colloidal gold preparation Au 198 in 11 cases of malignant tumours of the lung and pleura. By means of an apparatus designed for the purpose, injections were made in doses of 80—120 mc at a time, either at thoracotomy directly into the tumour or lymph node metastases, or in connection with pleural puncture into the pleural cavity. Following the injection the intensity and localisation of the radiation and the possible migration of the gold into the liver, spleen and urine were determined with a Geiger counter.

No side effects or complications ascribable to this method of treatment were seen after the therapy. In two cases insignificant amounts of gold were demonstrated in the urine. The liver function tests and the blood picture showed no changes.

Evaluation of the possible benefit from the use of radioactive gold is difficult on the basis of this small series of cases. However, in patients with pleural carcinosis there was a decrease in the amount of exudation and it was possible to prolong the intervals between aspirations. In the writers' opinion the advantage of gold therapy is a saving of time in inoperable or palliatively operated cases. The radiation effect starts already at operation, whereas ordinarily roentgen therapy cannot be instituted until after the patient has recovered from the surgical procedure.

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## EXPERIMENTS OF THE EFFECT OF RADIOACTIVE GOLD (AU 198) ON THE TRACHEAL TISSUE OF ANIMALS

by

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and MARTTI TURUNEN

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Concurrently with progress in atom physics, radioactive isotopes have gained increasing application in the service of medicine. Their use makes the local irradiation of tumour tissue possible. Consideration should therefore be given to the conditions under which the isotopes may have a more potent effect and their use would be more serviceable than that of radium and roentgen rays. The radiation properties of the radioactive colloidal gold preparation Au 198 have been considered suitable for this purpose. Already in 1945 Allen, Hempelman and Womack used radioactive gold in the colloidal form, and in 1947 Hahn, Goodell, Sheppard, Cannon and Francis described the use of Au 198 for the infiltration of tumours.

In postmortem examinations in cases of pleural and peritoneal carcinosis Kniseley and Andrews (1953) found fibrosis, petechiae and necrosis in the serous membranes. Gold was not always found to be present in the regional lymph nodes.

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<sup>1</sup> The late Professor P. E. A. Nylander, M.D., Head of the former First Surgical University Clinic, Helsinki, was greatly interested in the possibilities offered by isotopes in medicine. At his initiative, animal experiments were undertaken with radioactive gold (Au 198), which later also was applied to the treatment of inoperable cases of cancer of the lung. Professor Nylander participated actively in both the experimental and clinical studies. Unfortunately his untimely death on April 14, 1955, did not permit him to see the results of this work.

Regarding the localisation of Au 198 in the organism, Krabbenhoft (1954) stated that storage takes place in the reticulo-endothelial system, mainly in the liver, spleen and bone marrow and to a small extent in lung tissue. According to Vannotti, Fellingner and Vetter (1955) the gold is not localised in the tumour cells but in the histiocytes. It is bound to the reticular cells and moves with them.

In the opinion of Villaume (1954), the gold preparation in therapeutic doses produces necrosis or fibrosis in the lung tissue. The diseased lobe should be removed and the radioactive gold allowed to exert its effect on the local lymph nodes only. It appears, in fact, to have a tendency to pass from the lung or the pleura to the local lymph nodes. Thus Berg, Christophersen and Bryant (1955), after the bronchoscopic injection of radioactive gold under the mucosa of the dog trachea observed no pathological changes in the injected area but found an accumulation of the gold in the regional lymph nodes. Hahn, Rouser, Brummitt, Moorehead and Carothers (1952), in seeking to improve this effect, observed that in experimental animals the migration of the gold from the bronchial tree to the mediastinal lymph nodes was slow. Silver, on the other hand, moved at a considerably more rapid rate. For this reason they coated the radioactive gold with a silver capsule. Aurand (1955) has reported that Au 198 injected into the abdominal cavity entered the lymph nodes and the lymph channels. It was found as far as the supraclavicular lymph nodes.

The therapeutic possibilities for the use of isotopes in the treatment of malignant tumours are of great interest. It is well in this connection to know the effect of the substance employed and its distribution in the healthy organism. We have therefore considered it desirable to report the results of our animal experiments, although they are based on a limited material only. We have observed the effect of Au 198 on the tissues of the respiratory tract in particular.

#### EXPERIMENTAL

*Method and Material.* — Radioactive colloidal gold preparation Au 198 manufactured by the Radiochemical Centre, Amersham, was employed. The half-life of the preparation is 2.7 days, the particle size less than 30  $\mu$ , and the colloid is stabilised with gelatin. The  $\beta$ -ray energy is 0.97 MeV and that of  $\gamma$ -rays is 0.44

MeV. Owing to the short half-life, the radiation disappeared completely within two to three weeks. About 5 per cent of the activity remained after ten days.

Seventeen rabbits and twelve guinea pigs were used as experimental animals. The trachea was exposed under ether anaesthesia and an 1 cm wide strip of spongostan dipped in Au 198 was placed around the trachea. In the first experimental series the spongostan was moistened with 2 mc of gold solution. The animals in this series were eight rabbits and six guinea pigs, one-half of which were killed on the ninth day and the remainder on the sixteenth day. In the second series of four rabbits and two guinea pigs 20 mc of gold was used and the animals were sacrificed on the ninth day. The control series comprised two rabbits and two guinea pigs around whose trachea was placed a strip of spongostan wetted in the same manner with physiological saline. Into the pleural cavity of three rabbits and two guinea pigs were injected 6 mc of gold preparation. The animals were killed seven days after the injection.

The trachea with the paratrachea, tissue, and the thyroid, spleen and liver were saved. From the animals which had received pleural injections, specimens were taken of lung and pleural tissues. All the tissue samples were fixed in 10 per cent formalin. After embedding in paraffin, slices were cut at about 5  $\mu$ , stained by van Gieson and periodic acid methods, and examined under the microscope.

*Results.* — At macroscopic examination the operation wound of all the animals was found to have healed by first intention. The unresorbed strip of spongostan was in place around the trachea. No differences were observed between the controls and the animals treated with gold.

Microscopically the control animals showed in the tissues surrounding the unresorbed spongostan marked infiltration of inflammatory cells and some giant cells, the presence of which was ascribed to a foreign body reaction. There was no tissue necrosis. The changes observed were limited to the paratracheal tissue. The tracheal cartilage and mucosa were fully intact.

The animals having the paratracheal strip of spongostan dipped in gold preparation displayed the same histological changes as the control animals. No changes from the normal were observed in the cartilage, submucosa, mucosa and mucous glands. (Fig. 1.) There





Fig. 1. — Section of tracheal wall of a rabbit nine days after insertion against the trachea of a strip of spongostan moistened with 20 mc of Au 198. The mucosa is normal and the lymphoid tissue reveals no special features. The cartilage is healthy. Periodic acid Schiff stain.  $\times 120$ .

were no signs of Au 198 in the paratracheal lymph nodes (Figs. 2 and 3). However, the lymph nodes showed small necrosed areas which were disappearing; similar areas were not present in the controls. A further finding was thickening of the intima in some arterioles. The liver, spleen and thyroid of the experimental animals were normal.

Injection of radioactive gold into the pleural cavity produced no macroscopic changes in the pleura or the lungs. Microscopically there was an insignificant degree of thickening of the parietal and visceral pleurae.

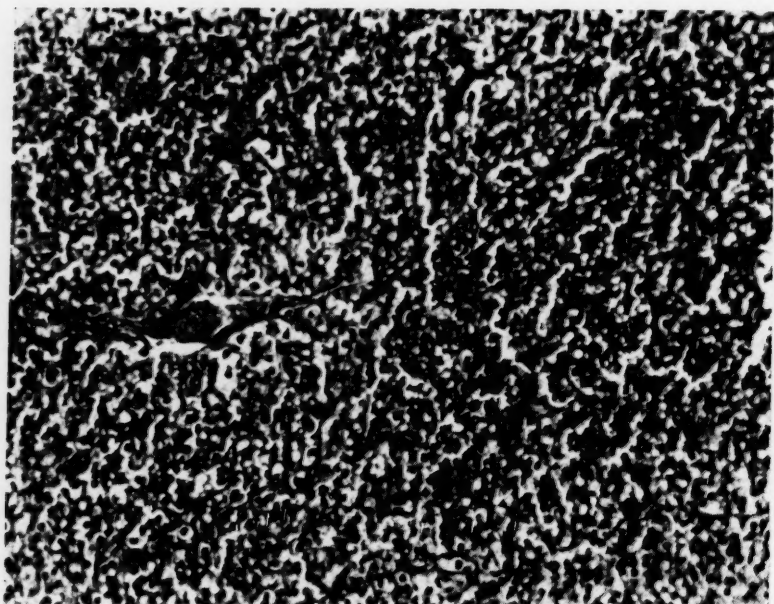


Fig. 2. — Section of a paratracheal lymph node from the same animal as in fig. 1, showing normal structure of the node. Van Gieson stain.  $\times 240$ .

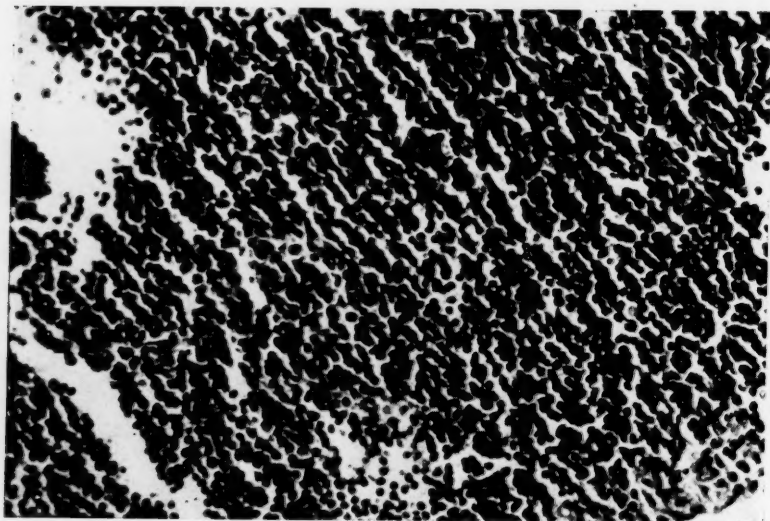


Fig. 3. — In higher magnification, disintegrating cells were seen in the lymph nodes as the only sign possibly ascribable to Au 198. Van Gieson stain.  $\times 560$ .

## DISCUSSION

Before a new preparation can be taken into clinical use its safety must be verified by animal experiments. In experiments made for this purpose we were unable to find any damage to the tissues caused by radiation from Au 198 placed in the paratracheal space of rabbits and guinea pigs. Berg, Christophersen and Bryant also failed to observe pathological changes in the tracheal tissue following the intrabronchial injection of gold into dogs.

In our animal experiments we observed but slight necrosed areas, which were about to disappear, in the paratracheal regional lymph nodes. This may have been due to the small dose of gold (2 mc) used in a part of the series, and to the killing of the animals at a time when any lymph node necroses which had developed were partly or wholly regenerated. It is furthermore possible that because of the spongostan strip the colloid was retained at the point of application for a longer time. From the clinical point it is important to know the route by which locally applied colloidal gold is distributed and resorbed from the area of application. A number of investigators (e.g., Andrews, Root and Kniseley, 1953; Berg, Christophersen and Bryant, 1955; Aurand, 1955) observed the accumulation of the gold into the lymph nodes of adjacent areas. However, this finding has not been confirmed in all the experiments made (Kniseley and Andrews, 1953; Vannotti, Fellingner and Vetter, 1955).

When injected into the pleural cavity of experimental animals the radioactive preparation did not produce even histologically demonstrable changes in the lungs or noteworthy reactive changes in the pleural tissue. A dose of only 6 mc of gold was employed in these experiments. Following the use of gold preparation Kniseley and Andrews (1953) observed at autopsy fibrosis, tissue necrosis and petechiae in the serous membranes.

## SUMMARY

In order to study the effect of the radioactive colloidal gold preparation Au 198 on respiratory tract tissues the writers placed strips of spongostan moistened with gold solution around the trachea of twelve rabbits and eight guinea pigs. The amounts of Au 198

used were 2 mc and 20 mc. A part of the experimental animals were killed on the ninth day and the remainder on the sixteenth day. The trachea with the paratracheal tissue and the thyroid, spleen and liver were examined histologically.

No macroscopic changes were observed in the organs of the experimental animals as compared with the controls. The microscopic examination revealed slight, disappearing necrosed areas in the paratracheal lymph nodes of the gold-treated animals. No alterations were present in the liver, spleen and thyroid gland.

In another experimental series 6 mc of gold preparation were injected into the pleural cavity of three rabbits and two guinea pigs. The animals were killed one week after injection and showed in the microscopic examination a slight thickening of the parietal and visceral pleurae.

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## EFFECT OF SUBPERITONEALLY INJECTED TWEEN 60 AND TWEEN 80 ON RATS<sup>1</sup>

by

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Several of the non-ionic lipo-hydrophilic Tweens have been shown to have strong tumor-promoting and co-carcinogenic properties in skin carcinogenesis in mice. When used alone they give rise, however, only to a few rapidly regressing benign tumors on the skin (1, 2). In an orientating experiment, designed to test the possible carcinogenicity of various Tweens, they were injected also subperitoneally into the wall of coecum and subperitoneally into the abdominal wall of rats. In this series one animal, treated with Tween 80, developed extensive sarcoma within eleven months. None of the other animals developed any tumors. The present experiment was planned to obtain information on whether the tumor in the orientating experiment was a coincidence or whether the Tween was the cause of the sarcomatous change.

### MATERIAL AND METHODS

53 albino rats of the same strain (Sprague-Dawley) as in the orientating experiment were used. The animals weighed 150 to 200 g at the begin of the experiment. Laparotomy was performed under aether anesthesia, and Tween 60 and Tween 80 were injected dropwise subperitoneally into the abdominal wall and subperi-

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<sup>1</sup> Supported by a grant from President J. K. Paasikivi's Foundation for Cancer Research.

toneally into the wall of coecum. Altogether 0.5 ml of Tween was injected in each animal. One of the animals died during the operation and two animals within 24 hours of the operation. There remained 25 rats treated with Tween 60 and 25 treated with Tween 80. They were kept on stock diet and given water ad libitum. Two of these animals died about half a year after the operation. The other animals were killed after a years observation period. Autopsy was performed and the sites of injection especially examined. The histological sections were stained with hematoxylin-eosin and hematoxylin-van Gieson.

#### RESULTS

As the results were identical in both groups (Tween 60 and Tween 80), they will be described together.

*Macroscopic Findings.* — No tumors were found. Intra-abdominal adhesions were seen in 16 of the 25 rats treated with Tween 60 and in 13 of the 25 rats treated with Tween 80. In three animals of the former group and two animals of the latter group the adhesions were extensive. In both groups approximatively half of the animals showed enlarged lymph nodes in the mesenterium of the coecum.

*Microscopic Findings.* — On the sites of injection the abdominal wall was edematous and sparsely infiltrated with lymphocytes and histiocytes. Scar formation was, however, seen only occasionally. In some cases small necroses were found. The enlarged mesenterial lymph nodes were congested and highly cellular. In some of them were small fibrotic foci seen. The wall of coecum showed in a few cases a strong and diffuse infiltration with especially lymphocytes. No malignant changes were found.

#### DISCUSSION

Tween 60 and Tween 80, when injected subperitoneally into the wall of the coecum and into the abdominal wall of rats, gave rise to inflammatory reactions which one year after the injection were manifest as lymphocytic infiltration and slight fibrosis at the site of injection. As the present experiment lasted twelve months, it seems likely that the extensive sarcomatous tumor observed in one

animal after eleven months in the orientating series was a coincidence and not due to Tween. The subcutaneous tissue of rats is known to be very sensitive to the influence of carcinogens. In the present experiment the Tweens were injected subperitoneally. The observation period was only one year. In this relatively short period the employed Tweens do not appear to induce any tumors at the site of treatment.

#### SUMMARY

In an orientating experiment in which the coecal walls of rats were injected with Tween 80, one animal developed large sarcomatous tumors within eleven months. The present experiment was performed to investigate in a larger series whether this tumor was related to the treatment with Tween or was a coincidence. Therefore in 25 rats 0,5 ml of Tween 60 was injected subperitoneally partly into the wall of coecum and partly into the abdominal wall. Twenty-five other rats were treated similarly with Tween 80. The animals were killed after twelve months. No malignant changes were seen in any animal. On the site of the injections chronic inflammatory alterations and slight fibrosis were observed. Intra-abdominal adhesions were common and sometimes extensive.

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## EXPERIMENTELL HERVORGERUFENE CHRONISCHE GASTRITIS

von

HARRY E. BLOMQUIST

(Eingegangen am 25. Juni 1956)

Im Zusammenhang mit einer früheren Arbeit habe ich (zusammen mit Setälä, Wangel und Holsti) chronisch atrophische Gastritis durch Einspritzung eines sklerosierenden Mittels in der Magenwand verursacht. Die vorliegende Arbeit ist ein Versuch, ein gleichartiges Ziel auf andere Weise zu erreichen.

### AUSGEFÜHRTE UNTERSUCHUNG

24 Meerschweinchen, deren Gewicht ungefähr 400 Gramm betragen hat, sind in Äthernarkose laparotomiert worden. Durch die vordere Wand des Magens und längs der Curvatura major in der hinteren Wand ist baumwollenes Nähgarn oberflächlich genäht worden. Die Tiere haben gewöhnliches Meerschweinchenfutter bekommen und sind nach folgenden Zeiträumen durch Chloroform getötet worden: nach einer Woche 2, nach zwei Wochen 2, nach drei 2, nach sechs 2, nach zwei Monaten 2, nach vier 3, nach sechs 4, nach acht 5 und nach neun 2. Sowohl der makroskopische als der mikroskopische Befund sind aufgezeichnet worden. Die verwendeten Gewebstückchen sind der behandelten Magenwand entnommen und unmittelbar in 10%iger Formalinlösung fixiert worden. Die Schnitte sind mit Hämatoxylin-Eosin und Hämatoxylin-van Gieson gefärbt worden.

## ERGEBNISSE

Nach einer Woche sind noch Oedem und Blutungen in der Magenwand vorhanden. Nach zwei kann ein Zusammenkleben der vorderen Magenwand mit der Leber beobachtet werden. Die Magenwand erscheint dicker als gewöhnlich. Lokale Nekrosen sind mikroskopisch festzustellen. An gewissen Stellen sind Veränderungen im Epithel in Form von kleinen Hohlräumen mit Pseudometaplasien (vergl. Schindler) zu sehen. Nach drei Wochen sind Verwachsungen mit der Leber, Rundzelleninfiltrate in der Submukosa, Hohlräume und helle Zellen im Epithel oberhalb der Muscularis mucosae zu erkennen. Die erwähnten Zellen erinnern an pseudopylorische Metaplasien. Nach sechs Wochen ist die Magenwand ziemlich viel dicker als gewöhnlich und die Verwachsung mit der Leber stark. Die Nähfäden sind an der Aussenseite des Magens kaum mehr zu sehen. In dem geöffneten Magen sind Endchen von dem Garn zu beobachten. Das Garn ist an mehreren Stellen entzwei. Nach zwei Monaten sind ähnliche Veränderungen wahrzunehmen. Nach vier ist der mit Nähfäden behandelte Teil der Magenwand geschrumpft, während der nicht behandelte Teil dilatiert ist. Nach sechs Monaten sind ziemlich viele Rundzelleninfiltrate in der Submukosa zu sehen. Nach 8—9 Monaten zeigen sich helle an Becherzellen erinnernde Zellen. Endchen von Nähgarn sind noch wahrnehmbar. Bei den zwei neun Monate nach der Behandlung getöten Meerschweinchen sind die Anzahl der Mastzellen in der Submukosa des Magens mit Hilfe der von Hjelmman (1954) empfohlenen Methode bestimmt worden. In der Volumenheit — 1 cmm — sind durchschnittlich 7093.9 Mastzellenkerne gefunden worden.

Der nicht behandelte Teil der Magenwand scheint normal zu sein.

## BESPRECHUNG DER ERGEBNISSE

Die Durchnähung der Magenwand mit baumwollenem Nähgarn hat lokale Veränderungen verursacht, die anfangs recht gering sind, aber allmählich stärker werden. Anfangs sind sie meist in der Serosa lokalisiert und verursachen eine Verwachsung mit der Leber. Die Magenwand wird allmählich dicker und schrumpft. In sämtlichen Schichten des Magens sind Rundzelleninfiltrate als Zeichen

einer Entzündung wahrnehmbar. Auch das Epithel zeigt gewisse Veränderungen. Zuerst treten kleine Hohlräume mit Pseudometaplasien auf, dann Zellen, die an pseudopylorische Metaplasien erinnern und nach acht bis neun Monaten solche, die Becherzellen ähneln.

Die Nähfäden sind nach ungefähr sechs Wochen entzwei, die Endchen ragen aus der Mucosa hervor. Wahrscheinlich ist dies als eine Wirkung der Salzsäure des Magens aufzufassen.

Die neun Monate nach der Operation gefundene Anzahl der Mastzellen ist im Vergleich mit dem normalen Befund erhöht (vergl. Blomquist 1956). Janes und McDonald (1948) haben gezeigt, dass die Anzahl der Mastzellen in Präparaten mit Gastritis hoch ist. Blomquist (1956) hat dieselbe Beobachtung bei auf andere Weise experimentell hervorgerufener Gastritis gemacht.

Die festgestellten Veränderungen der Magenschleimhaut sind recht gering. Sie sprechen jedoch für eine chronische Gastritis. Die verwendete Methode kann also zur Hervorrufung einer solchen gebraucht werden. Die Veränderungen sind aber lokalisiert und entstehen recht langsam. Die Methode ist darum ziemlich zeitraubend.

#### ZUSAMMENFASSUNG

Die Durchnähung der Magenwand mit baumwollenem Nähfaden verursacht einen chronisch inflammatorischen Reiz, der auf alle Schichten der Magenwand zu wirken scheint. In der Mukosa entsteht eine lokalisierte chronische Gastritis.

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## SHORT-TIME EFFECT OF BENZPYRENE ON EOSINOPHILS

OBSERVATIONS ON THE SHORT-TIME EFFECT OF ORALLY ADMINISTERED BENZPYRENE UPON THE NUMBER OF EOSINOPHILIC CELLS IN THE ALIMENTARY CANAL, BONE MARROW AND BLOOD OF THE RAT<sup>1</sup>

by

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(Received for publication July 24, 1956)

Although there are extensive studies on eosinophilic cells, investigations on tissue eosinophilia, especially from the quantitative point of view, are relatively scarce. The number of eosinophils in the blood and various tissues has simultaneously been observed in few studies only.

It has been established since long that the mucosa of the alimentary canal in starved animals contains less eosinophils than usual (7, 1, 13). The treatment with ACTH has increased the number of eosinophils in the submucosa of the small intestine and the colon (5, 11). A similar phenomenon has been observed in the submucosa of the small intestine by injecting protein-free extract from ascaris and histamine (20). The administration of pilocarpine and atropine, as well as vagotomy, have also increased the number of eosinophils in the lamina propria of the glandular stomach of the rat (18). Besides the eosinophils in the blood, a marked increase in the number of tissue eosinophils has generally been established in connection with anaphylactic reactions (14, 15).

<sup>1</sup> This investigation is supported by a grant from the Damon Runyon Memorial Fund (DRG-291-A).

Based on the previous investigations in our laboratory (18), the purpose of this work was to study the effect of a toxic agent, locally irritating the alimentary canal, on eosinophils. From carcinogenic hydrocarbons, benzpyrene was selected for this purpose.

#### METHODS

The present investigation was carried out on about 7-month-old white male and female rats, which were all kept under the same conditions. They were fed ad libitum with a mixture of oatmeal, ryeflour, oat bran and wheat bran 22.25 per cent each, casein 4.45 per cent, calcium lactate 1.0 per cent, yeast 2.2 per cent, salt 0.75 per cent, butter 2.2 per cent and cod-liver oil 0.4 per cent. Before the experiment the rats were starved for two days.

Some of the control animals were untreated, some were administered a dose of 1 ml of olive oil. These controls were necessary because benzpyrene was solved in olive oil in the main experiments, and we wanted to check the possible effect of olive oil, *per se*. One ml of olive oil containing 0.5 per cent (5 mg) benzpyrene was cannulized with a rubber cannule into the stomach.

The rats were killed by decapitation in groups  $\frac{1}{2}$ , 1, 4 or 12 hours after cannulization. Blood samples were taken by cutting the end of the tail before cannulization and just before killing. The eosinophils in the blood were stained with phloxin in propylen-glycol. The cells were counted in Bürker's and Fuchs-Rosenthal's cells. All groups were cannulized so that they could be decapitated at the same time of day (at 7.00—8.00 P.M.). Diurnal variations were thus avoided. Immediately after decapitation the femur was severed. A smear was prepared from the bone marrow and stained with Giemsa's solution. The percentage of eosinophils was calculated from 500 nuclear cells of the marrow.

A specimen from the stomach from the site of the great curvature including about 5 mm of the forestomach and the whole glandular stomach to the pylorus was removed, attached to a piece of cardboard and fixed in Bouin's fixative for 12 hours. A specimen from the duodenum was taken at a distance of about  $1\frac{1}{2}$  cm from the pylorus. A specimen from the end of the small intestine was removed at about 9 cm, and from the colon at about 6 cm before the caecum. All the specimens were treated in the same way. After embedding

in paraffin, the specimens were sectioned at about  $3\ \mu$  and stained with hemalum-eosin.

For the calculation of the tissue eosinophils a Zeiss micrometer-eyepiece  $5\text{ mm} \times 5\text{ mm}$ , a Reichert ocular 4. and a Reichert 18 b  $1/12''$  objective were used, with a magnification of about 900. Because the eosinophils of the glandular stomach were most abundant in the lamina propria, the count was commenced at the border between the forestomach and the glandular stomach. The micrometer-eyepiece was focussed tangential the crypts of the glands, and the eosinophils falling within a rectangle of  $5\text{ mm} \times 1\text{ mm}$  were counted. A count was made of a total of 30 such rectangles, corresponding to an area of 0.05 sq.mm in the section.

The exact count could not be made in the duodenum and the ileum because of the thinness of the lamina propria. Besides, the eosinophils being relatively scarce in the lamina propria adjacent to the lamina muscularis mucosae, the count was made from the villi in which the cut was in the middle, *i.e.*, the count could be made from the points of the villi to the lamina muscularis mucosae. As previously, 30 rectangles were counted. In the colon, the rectangle was placed tangentially to the bottoms of the crypts. Likewise, 30 rectangles were counted.

#### RESULTS

A marked increase in the number of eosinophils was noted in the stomach at  $1/2$ , 1 and 4 hours after the treatment with benzpyrene (Table 1 and Fig. 1). At 12 hours the values obtained did not differ from the normal. The administration of olive oil, *per se*, also brought about a slight rise at  $1/2$ —4 hours, the differences, however, not being marked as compared with the controls. A great number of eosinophils was encountered at the border between the forestomach and the glandular stomach, while only few or none of them were found inside the forestomach. In the glandular stomach the eosinophil count showed a moderate dispersion. No difference in the numbers of eosinophils between various groups was observed in the forestomach.

No clear effect of benzpyrene and olive oil was established in the duodenum, ileum and colon. In the duodenum and ileum most eosinophils were found around the points of the villi. Many eosinophilic formations were encountered in the colon.

TABLE 1

COUNT OF EOSINOPHILIC CELLS IN ALIMENTARY CANAL AND BONE MARROW

Experimental Details	Number of Rats	Mean of Eosinophilic Cells $\pm$ SD					Bone Marrow %
		Stomach	P	Duodenum	Ileum	Colon	
Controls	11	64.0 $\pm$ 14.7		79.5 $\pm$ 22.9	76.3 $\pm$ 27.1	90.1 $\pm$ 16.4	11.0 $\pm$ 1.6
Olive oil							
$\frac{1}{2}$ h	5	92.8 $\pm$ 23.6		81.4 $\pm$ 19.6	73.6 $\pm$ 17.1	93.4 $\pm$ 23.6	10.4 $\pm$ 0.7
1 h	4	91.0 $\pm$ 17.0		87.8 $\pm$ 32.0	65.5 $\pm$ 16.7	79.5 $\pm$ 4.6	12.0 $\pm$ 1.4
4 h	5	78.0 $\pm$ 22.1		78.6 $\pm$ 22.1	86.4 $\pm$ 35.3	93.0 $\pm$ 28.2	9.0 $\pm$ 1.9
12 h	4	53.8 $\pm$ 10.5		85.0 $\pm$ 34.5	97.5 $\pm$ 19.8	82.3 $\pm$ 26.6	13.2 $\pm$ 1.6
Benzpyrene							
$\frac{1}{2}$ h	5	112.8 $\pm$ 10.0	0.01	87.0 $\pm$ 10.3	69.8 $\pm$ 17.2	89.2 $\pm$ 17.2	10.1 $\pm$ 1.9
1 h	5	109.8 $\pm$ 28.0	0.05	88.4 $\pm$ 16.6	86.2 $\pm$ 17.0	101.8 $\pm$ 39.5	11.5 $\pm$ 1.4
4 h	5	114.6 $\pm$ 17.7	0.025	92.0 $\pm$ 8.8	84.0 $\pm$ 13.2	101.2 $\pm$ 30.7	8.9 $\pm$ 2.1
12 h	5	68.2 $\pm$ 7.3		76.8 $\pm$ 18.0	81.2 $\pm$ 5.9	72.0 $\pm$ 8.2	12.4 $\pm$ 0.6

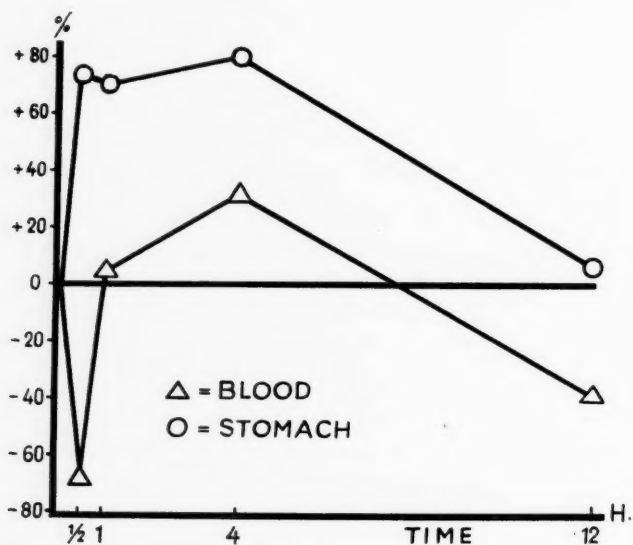


Fig. 1. — Effect of benzpyrene on the eosinophils of the blood and the stomach.



TABLE 2  
COUNT OF EOSINOPHILIC CELLS IN BLOOD

Experimental Details	Number of Rats	Mean of Eosinophils		Alteration %
		Before	After	
Normal	49	695.5		
Olive oil				
$\frac{1}{2}$ h	5	895.8	717.0	— 20
1 h	4	924.0	679.3	— 26.5
4 h	5	746.2	281.8	— 62.2
12 h	4	291.8	178.5	— 42.2
Benzpyrene				
$\frac{1}{2}$ h	5	1082.4	381.6	— 65.0
1 h	5	701.8	718.0	+ 2.3
4 h	5	502.6	650.2	+ 29.0
12 h	5	675.4	380.6	— 43.5

Great individual differences were observed in the numbers of eosinophils in the blood. The highest value in the group of 49 rats before cannulization was 2519 cells per 1 cu.mm, and the lowest 33 cells per 1 cu.mm. The mean obtained was 695.5 cells per 1 cu.mm. Because of the variations of individual normal values, each group was taken as a unit and the mean of the eosinophil counts was compared before and after the experiment (Table 2 and Fig. 1). The treatment with olive oil resulted in the diminution in the number of eosinophils, the minimum being at 4 hours. As compared with olive oil, benzpyrene brought about a much greater decrease at half an hour. At 1 hour, the initial and final values were about the same, while a marked increase was observed at 4 hours. The decrease at 12 hours was approximately the same in both cases.

In the bone marrow, the mean of 11 controls was 11 per cent of the nuclear cells of the marrow. There was no difference between the effects of benzpyrene and olive oil, but as compared with the controls slight variations occurred at different hours (Table 1 and Fig. 1).

#### DISCUSSION

The great number of eosinophils in the stomach might be due to the fact that the rats used in the present study were older than those previously used in our laboratory (18). It has been observed

that the eosinophils in the blood of rats increase with the age (17), the mean of 5-month-old rats being 556 cells per 1 cu.mm. In the present study, the corresponding mean of 7-month-old rats was 695.5 cells per 1 cu.mm. Eosinophils have been found in the intestine of the adult rabbit though none of them have been observed in the embryo (1). It was noted during these experiments that the control animals with many eosinophils in the blood also displayed a great number of them in the submucosa of the alimentary canal.

In fact, the changes in the eosinophilia of the blood brought about with benzpyrene correspond to those described in connection with the «alarm reaction» (3, 6). The maximum of eosinophils at 4 hours in the present study does not suggest the effect of ACTH and adrenocorticoids (19). The changes brought about with olive oil, on the contrary, might be explained through them (19), supposing that, *e.g.*, wriggling at cannulization should increase the secretion of the hormones.

In the blood of the rats treated with benzpyrene the number of eosinophils was diminished at half an hour, while a marked increase was noted in the stomach (Tables 1 and 2 and Fig. 1). This could be interpreted, *e.g.*, as follows.

- 1) The eosinophils of the blood proceeded into the stomach (20).

- 2) Rapid development of eosinophils in the stomach itself took place simultaneously with the decrease of the number of eosinophils in the blood (5).

The fact that the number of eosinophils in the blood remained the same at 1 hour and slightly increased at 4 hours could be explained by the compensation, or even hypercompensation, of the bone marrow for the decrease of eosinophils perhaps originating in the blood (9). Sufficiently clear reflection in the number of eosinophils in the bone marrow was not, however, observed though slight differences occurred at different hours, and the values resembled those obtained by Esselier with the administration of ACTH (4). The differences in the present study were, however, still smaller. The decrease of eosinophils in the bone marrow with various intestinal worm preparations was brought about by Homma in connection with the tissue eosinophilia (9). During eosinopenia in the blood, eosinopenia in the peritoneal fluid, too, has been produced with adrenal hormones (8, 12, 16). On the other hand, Speirs succeeded in increasing the number of eosinophils with various

agents simultaneously in the blood and the tissues (16). In the present study, a similar phenomenon was established with benzpyrene at 4 hours (Fig. 1). The decrease of eosinophils in the blood at 12 hours may, perhaps, be due to diurnal variations (10). (The first count was made at 9.00 A.M. and the second at 9.00 P.M.).

Extramedullary eosinopoiesis has been reported to take place on the mucous membrane of the intestine of the dog, and the «eosinophilization» of epithelial cells has been given as evidence of this (5). This phenomenon was not observed in the intestine of the rats used in the present study. On the other hand, eosinophilic juvenile forms were encountered in the entire alimentary canal.

In the small intestine of the cat (2) and the rabbit (1), most eosinophils have been observed round the base of the villi. In these experiments, most of them occurred round the points of the villi and were situated on either side of the lymphatic vessel in the middle. The majority of the eosinophils in the villi were myelocytes, while in the sites adjacent to the lamina muscularis mucosae forms with horseshoe-shaped nuclei were most common, though myelocytes, metamyelocytes and forms with segmented nuclei also occurred. The nuclei of the eosinophils round the villi seemed to stain darker than usual.

#### SUMMARY

The observations were made on the short-time local effect of benzpyrene on the eosinophilia of the alimentary canal and on the eosinophils of the blood and the bone marrow.

In the stomach of 11 rats starved for two days the mean of the eosinophil counts was  $64.0 \pm 14.7$  per 0.05 sq.mm. A significant increase up to  $114.6 \pm 17.7$  was brought about with benzpyrene at  $\frac{1}{2}$ —4 hours. Olive oil had no marked effect (Table 1).

No great changes in the numbers of the eosinophils in the duodenum, ileum and colon were observed in different groups.

In the small intestine, the majority of eosinophils were found round the points of the villi. Special attention was paid to the localization of different eosinophilic forms.

In the blood of 49 rats starved for two days the mean was 695.5 cells per 1 cu.mm. The number of the eosinophils in the blood was compared before and after the experiment. The administration

of benzpyrene brought about the decrease of 65 per cent at half an hour, no change at 1 hour, the increase of 29 per cent at 4 hours and the decrease of 43.5 per cent at 12 hours, while olive oil caused decrease at all given hours. The decrease at the lastmentioned hour may partly be due to diurnal variations.

In the bone marrow of 11 control rats the mean of the eosinophil counts was 11 per cent of the nuclear cells. No marked differences were observed in any groups.

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## A METHOD FOR THE DETERMINATION OF THE PENICILLIN LEVEL IN VARIOUS PARTS OF THE RESECTED LUNG

by

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The purpose of the experiments described in the present publication was to develop a method for the determination of the penicillin level in various parts of a resected lung, for instance a tuberculous one. From the available literature we have only found a study made with lungs of normal dog concerning the absorption of penicillin into the lung tissue (2).

### MATERIAL AND METHODS

*The patients* were persons with pulmonary tuberculosis, both males and females aged 32—48 years and weighing 64—72 kilograms. They had all received antituberculous drug treatment for some length of time and had then been admitted to a surgical hospital for pneumectomy or lobectomy.

*Dosage of Penicillin.* — A few hours before the surgical operation the patients were given an intramuscular injection of 600,000 IU of procaine penicillin in aqueous suspension. Since this dosage appeared to be too low for the method of penicillin assay used, it was increased to 2 million of sodium G penicillin in aqueous solution, given intramuscularly two hours before operation.

*Preparation of Lung Samples.* — The lung or portion of lung resected at operation was thoroughly frozen in the deep-freeze at  $-20^{\circ}\text{C}$ .

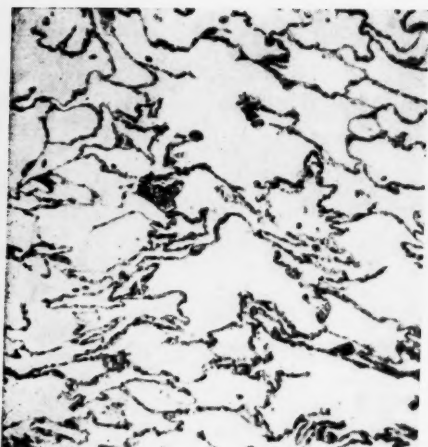


Fig. 1. — Normal lung.

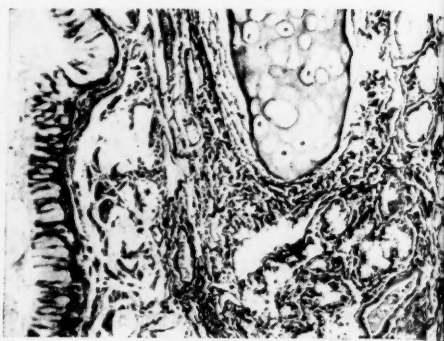


Fig. 2. — Normal bronchus.

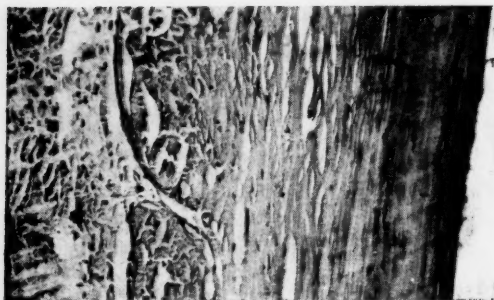


Fig. 3. — Pleural thickening.

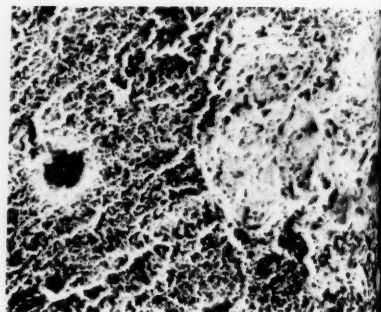


Fig. 4. — Tuberculous lymph node.

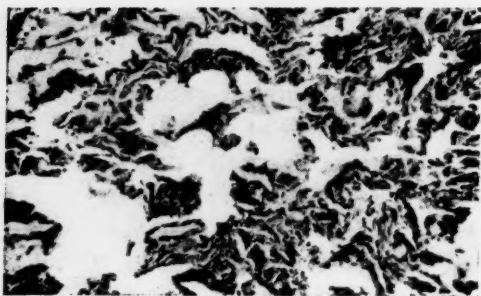


Fig. 5. — Fibrosis.

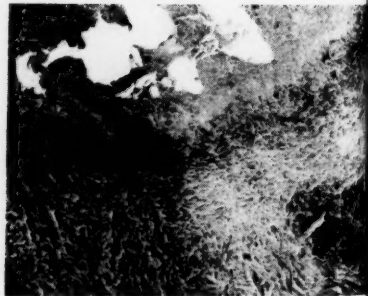


Fig. 6. — Cavity wall.



Samples were taken from selected portions of the frozen lung for the penicillin assay and microscopic examination. The taking of samples was facilitated by allowing the lung to defreeze for 10—15 min. It was then cut into slices about 1 cm thick. The large blood vessels, bronchi and tuberculous processes of different kinds were visible in the cut surfaces. On basis of the macroscopic examination, cylindrical samples 0.8 cm in diameter were taken from different parts of the organ with a cork borer. The samples were cut longitudinally into two halves, one of which was used for histological examination and the other for the penicillin. For the latter purpose the sample was weighed, ground and extracted with 1 ml of normal human serum.

For determination of the serum penicillin level a blood sample was taken during the surgical operation.

The penicillin assay method used was the «*Sarcina lutea*, cup plate method» (1), with minor modifications.

#### RESULTS

The first test series revealed that after administration of 600,000 IU of procaine penicillin the extract made from a normal portion of the lung contained 0.03 IU of penicillin or even less. Since 0.03 IU/ml was the minimal value in the penicillin assay technique used in these tests, it was difficult and even impossible to perform comparative tests with this penicillin dosage.

When the patient received 2 million IU of sodium G penicillin the sample extracts were found to contain very considerable amounts of penicillin. Table 1 shows the values obtained from the samples from four patients. Prior to these patients, seven lungs had been studied, but the results were unsatisfactory because of deficiencies present at first in the technique. The values in the table are expressed in units of penicillin per gramme of lung tissue in the sample. As will be seen from this table, moderate amounts of penicillin were demonstrated by this method in all the tuberculous processes, even within the foci of caseous necrosis. The first values listed in the table are the penicillin concentrations in the serum. These varied greatly, as was to be expected, since the blood samples were taken at different times after the penicillin administration owing to the varying duration of the operative procedures, and



since the penicillin concentration in the blood falls abruptly within a few hours after the injection of an aqueous solution of sodium G penicillin.

In table 2 the values for samples from the four patients are grouped according to type or location of sample, and the serum/tissue ratio of the penicillin concentrations are given. This serum/tissue ratio is of the same order of magnitude in all the patients with the exception of patient No. 3, in whom the serum concentration was only 1 IU/ml and the ratio was low in all the samples. The serum sample in this case was taken an exceptionally long time after the penicillin administration, and the penicillin had almost disappeared from the serum although still present in the tissues in moderate amounts. In other patients the ratio ranged from 6 to 50 according to the type of tissue and the tuberculous process. In other words, there was 6–50 times less penicillin in the tissues than in the serum, but with the method employed it was possible to demonstrate the presence of penicillin in all the tuberculous lesions.

TABLE 1

PENICILLIN CONCENTRATION IN THE RESECTED LUNGS OF FOUR PATIENTS, CALCULATED IN IU OF PENICILLIN PER GRAMME OF LUNG TISSUE IN SAMPLE. TWO HOURS BEFORE THE SURGICAL OPERATION EACH PATIENT RECEIVED 2 MILLION IU OF SODIUM G PENICILLIN IN AQUEOUS SUSPENSION BY INTRAMUSCULAR INJECTION

Patient 1		Patient 2		Patient 3		Patient 4	
Sample	Penicillin IU/g	Sample	Penicillin IU/g	Sample	Penicillin IU/g	Sample	Penicillin IU/g
Serum .....	20	Serum .....	12	Serum .....	1.0	Serum .....	5.0
Fibrosis ....	2.7	Wall of cavern..	0.7	Tuberculous ..	0.7	Wall of cavern ..	0.2
Tuberculous lymph gland	1.0	Tubercles ....	1.2	lymph gland ..	1.2	Fibrosis ....	0.8
Tuberculoma+ fibrosis ....	5.0	Caseation .....	0.7	Pleura .....		Normal bronchus ..	0.1
Wall of cavern ..	2.0	Bronchus and peribronchial tbc.	0.9	Hypertrophic pleura .....	1.8	Content of cavern .....	0.3
Carnification ..	1.0	Fibrosis .....	1.6	Normal lung tissue .....	2.1	Normal lung tissue ....	0.3
Tubercles ....	3.0	½ normal + ½ fibrosis ....	2.0	Tbc-pneumonia ..	1.3	Normal bronchus ..	0.2
Fibrosis ....	0.7	Tubercles .....	1.8	Tbc-pneumonia ..	0.5	Normal lung tissue ....	2.4
		Caseation .....	1.6	Caseation .....			
				Normal lung tissue .....	2.4		

TABLE 2

PENICILLIN CONCENTRATIONS IN TUBERCULOUS PROCESSES IN THE LUNGS OF FOUR PATIENTS, GROUPED ACCORDING TO TYPE OF PROCESS AND EXPRESSED AS THE RATIO BETWEEN THE PENICILLIN CONCENTRATION IN THE SERUM AND IN THE TISSUE

	Patient 1		Patient 2		Patient 3		Patient 4	
	IU/g	Serum/ tissue ratio	IU/g	Serum/ tissue ratio	IU/g	Serum/ tissue ratio	IU/g	Serum/ tissue ratio
Normal lung (Fig. 1) .....					2.1 2.4	0.5 0.4	0.3 0.3	17 17
Normal bronchus (Fig. 2) ....			0.9	13			0.1 0.2	50 25
Pleural thickening (Fig. 3) ....					1.8 1.2	0.6 0.8		
Tuberculous lymph node (Fig. 4)	1.0	20			0.7	1.4		
Fibrosis (Fig. 5) .....	2.7 0.7	7 29	1.6	8			0.8	6
Tubercles .....	3.0	7	1.8 1.2	7 10				
Cavity wall (Fig. 6) .....	2.0	10	0.7	17			0.2	25
Caseous necrosis .....			0.7 1.6	17 8	0.5	2		
Cavity contents .....							0.3	17

## DISCUSSION

A series of four patients is too small to permit any definite conclusions regarding the usual distribution of penicillin in the various parts of a tuberculous lung. However, in the light of the results obtained it seems obvious that the method employed, in which the samples are taken from various parts of deep-frozen lung, gives comparatively reliable data on the distribution of the penicillin in the different parts of the lung and in the tuberculous lung processes of different types. The drawing of conclusions is also difficult because samples of processes of the same type cannot always be obtained from several persons and because macroscopic examination cannot always assure that samples taken even from the same lung

will contain only one type of tissue. Furthermore, processes of the same type may be differently vascularised in different parts of the lung and in different persons. During these experiments the same penicillin preparation was used throughout, but, as has been previously demonstrated by Savolainen and Tommila (3, 4, 5), not only the products of different manufacturers but also different batches from the same may give different serum penicillin concentrations.

Penicillin determinations will not, of course, indicate how other antibiotic and chemotherapeutic drugs used in the treatment of tuberculosis will be distributed in the different parts of a tuberculous lung. However, a method which follows the same principles should be applicable in the determination of, for instance, the streptomycin content. Studies on this point are in progress.

#### SUMMARY

A method is presented for the determination of the penicillin concentration in different parts of a resected lung. The resected lung is deep-frozen and samples for penicillin assay and histological examination are taken from the frozen lung.

By the method developed in the present study it was found that after the intramuscular injection of 2 million IU of sodium G penicillin in aqueous solution into a patient with pulmonary tuberculosis two hours before the surgical operation, moderate amounts of penicillin were demonstrable in all parts of the resected lung and within the tuberculous lesions.

The results of these determinations and the application of the same method to the examination of the distribution of other antibiotics and chemotherapeutic drugs in various disease processes of the lung are discussed.

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## COXSACKIE ANTIBODIES IN DOMESTIC ANIMALS AND NORWAY RATS

by

PIRKKO POHJANPELTO and P. VUOPIO<sup>1</sup>

(Received for publication September 4, 1956)

The idea that Cocksackie viruses have some animal hosts besides man was aroused by the investigation of O'Connor and Morris (1, 2). They were able to isolate a Cocksackie virus, type A4, from the blood of a wild rabbit and they found neutralizing antibodies against Cocksackie types A1, A3, A4, and A7 in several rabbits, one marmot and one fox. The presence of neutralizing antibodies has also been established in apes imported directly from Africa (3). The present investigation is a contribution to the search for animal reservoirs for Cocksackie viruses.

### MATERIAL AND METHOD

*Sera* — Swine, cow, sheep and horse sera were collected in a slaughterhouse in Helsinki, where animals were brought from the southern part of Finland. The rat sera were from wild Norway rats captured alive in Helsinki.<sup>2</sup>

*Mice* — 1—2 days old Albino Swiss mice were used.

*Neutralization Test.* — All sera were inactivated 30 min. at 56 C. Serum-virus mixtures were allowed to stand for one hour at room temperature before injection into the mice. If there were not enough litters for all the solutions made, the rest of the solutions were placed in a refrigerator at -20°C and injected some days

<sup>1</sup> With the technical assistance of Miss Pirkko Forsgrén.

<sup>2</sup> The rat sera were kindly placed at our disposal by Dr. A. Salminen to whom we are greatly indebted.

later when new litters were available. Every solution was injected into one litter of mice containing at least 5 young. The mice were observed daily for ten days. If during this time half the mice or more had disappeared (cannibalism) the solution was tested once more. Every titration included virus titration with two litters. To save mice, five sera were pooled together so that the final dilution of each serum would be 1/10. If a pool showed virus inhibition the sera contained in the pool were tested individually. The cow sera, however, were not examined individually.

## RESULTS

40 swine sera, 35 cow sera, 6 sheep sera and 2 horse sera were tested for the presence of A10 neutralizing antibodies (Table 1).

TABLE

	A10		A4		B1	
	No. Tested	No. Positive	No. Tested	No. Positive	No. Tested	No. Positive
Swine ..	40	17				
Rats ....	40	1	40	0	30	0
Sheeps ..	6	0				
Horses ..	2	0				
Cow sera pools (each containing 5 sera) ..	7	4				

17 of the swine sera, i.e. 43%, could in dilution 1/4 neutralize 100 ID<sub>50</sub> or more of the virus. 5 of them, i.e. 13%, could neutralize more than 100 ID<sub>50</sub>. From 7 cow serum pools, each containing 5 sera, four neutralized 100 ID<sub>50</sub> of virus. The sera of one cow serum pool were tested individually and one of them neutralized in dilution 1/4 100 ID<sub>50</sub> of virus. None of the cow sera could in dilution 1/10 neutralize more than 100 ID<sub>50</sub> of virus. The 6 sheep sera and 2 horse sera showed no neutralizing capacity.

40 rat sera were tested for the presence of A4 and A10 neutralizing antibodies and 30 rat sera for the presence of B1 neutralizing antibodies. Only one of them contained A10 antibodies. This serum could in dilution 1/50 neutralize 100 ID<sub>50</sub> of virus. The same serum did not show any neutralizing capacity against A4 and B1 viruses.

## DISCUSSION

Sera of the same animal group showed different neutralizing capacities. Thus, the neutralizing capacity does not seem to be a specific ability of a species. All sera were inactivated before testing; therefore there is no question of nonspecific, thermolabile, virus activating substances. It is possible that the animals with Coxsackie antibodies had previously had Coxsackie infection, but it is also conceivable that they had been infected with some other micro-organism antigenically related to Coxsackie. Coxsackie virus isolations would be of decisive importance.

It should be mentioned that poliomyelitis antibodies have also been found in domestic animals, but in spite of many attempts poliomyelitis viruses have not been isolated from them (4). If it could be shown that the animals concerned are Coxsackie or poliomyelitis virus carriers, it would do much to clarify the epidemiologic patterns of these viruses.

## SUMMARY

Coxsackie A10 neutralizing antibodies have been found in 17 out of 40 swine sera, in 4 out of 7 cow serum pools each containing 5 sera and in one out of 40 rat sera. 6 sheep sera and 2 horse sera did not have these antibodies.

Coxsackie A4 neutralizing antibodies were not found in 40 rat sera nor B1 neutralizing antibodies in 30 rat sera.

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## CHROMATOGRAPHIC STUDIES OF AMINO ACID CONSTITUENTS OF PURE CANINE DUODENAL JUICE

by

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(Received for publication September 7, 1956)

Duodenal analyses have been used for clinical purpose and in this connection also the amino acid composition of the mixed juice has been analysed (4). Pure duodenal juice of dog has been available for us and we have performed chromatographic analyses on its amino acid composition. The results will be reported here since to the authors knowledge no similar analyses have been made on pure, native duodenal juice.

### MATERIAL AND METHODS

The duodenal juice was collected from dogs prepared with chronic duodenal pouches. Such pouch was made from the first segment of duodenum beginning at the pylorus and ending at the junction of the common bile duct with the duodenum. This part of duodenum was excised and brought through a stap wound through the abdominal wall with its original blood and mesenteric nerve supply. The continuity of the alimentary canal was restored by gastroduodenostomy.

These pouches produce alkaline and thick mucus containing juice, which can be directly collected into test tubes. It should be pointed out that the bile and pancreatic juice are not secreted from these pouches.

Precautions were made to prevent the enzymatic degradation of the mucus which has previously been studied by one of us (3).

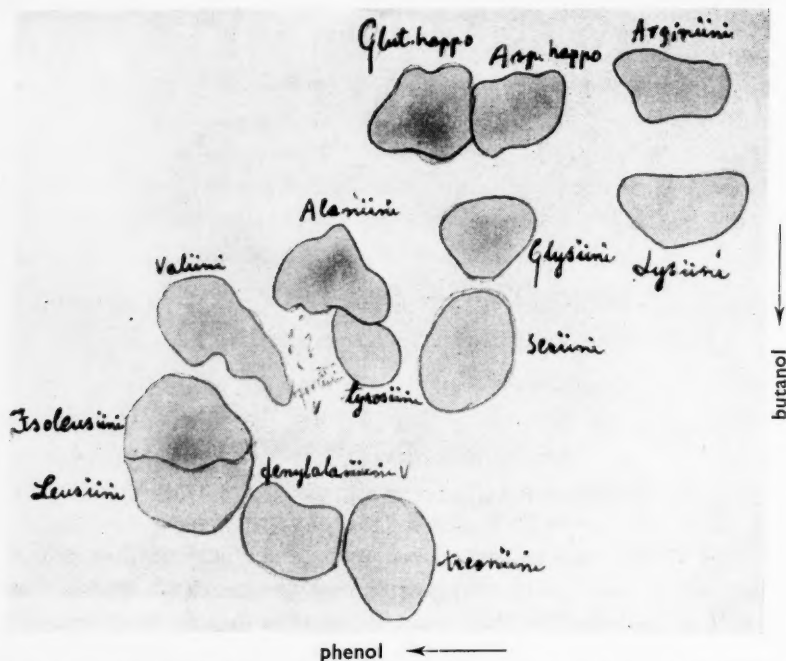


Fig. 1. — Two-dimensional chromatogram of pure canine duodenal juice obtained from duodenal pouches.

The juice was therefore collected directly into the tubes placed in ice and stored under similar conditions. The material was collected from 6 different dogs.

The juice was first hydrolysed with concentrated HCl-acid according to the procedure described by Dent (2). The two-dimensional paper chromatographic procedure was employed in the study. Phenol and n-butanol were used as the solvents and Whatman No. 1 filter paper for the separation of the amino acids. The color development was achieved by spraying the papers with 0.1% ninhydrin solution and the chromatograms dried in dark at room temperature for 24 hours. The separation of cystine and cysteine was performed with sodium-nitroprussid solution according to Toennies et al. (5).

Control chromatograms using pure amino acid solutions were performed. Altogether some 150 runs were made and the results for known amino acids were fairly constant and agreed closely to those reported in the literature (1).

## RESULTS

Some 50 analyses were made and the chromatograms obtained from the juice of the 6 different dogs were all similar. This was an interesting observation for us since two of the pouches had been in use for 3 years already.

The total chromatogram of the duodenal juice is illustrated in figure 1.

It appears that the amino acid chromatograms of duodenal juice lacks signs of striking interest but contains those amino acids present in many other similar biological products.

## SUMMARY

Chromatograms were performed of pure duodenal juice collected directly from duodenal pouches in dog. The following 13 amino acids were detected:

- 1 Arginine
- 2 Aspartic acid
- 3 Glutamic acid
- 4 Lysine
- 5 Glysine
- 6 Alanine
- 7 Serine
- 8 Valine
- 9 Tyrosine
- 10 Iso-Leucine
- 11 Leucine
- 12 Phenylalanine
- 13 Threonine

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## FACTORS IN SERUM AFFECTING THE GROWTH OF HELA CELLS<sup>1</sup>

by

ERKKI SAXÉN and KARI PENTTINEN

(Received for publication September 8, 1956)

In a preliminary paper (4) the present authors reported that, when compared with cultivation in nonheated sera, the cultivation of HeLa cells in inactivated pools of human sera caused higher cell counts. This led us to assume either the destruction of a cell growth controlling system in the serum or the production of a cell growth stimulating condition by the inactivation of the serum. Since the possible existence of a growth controlling system in serum seemed to us to be of great potential value, the experiments were extended. In investigating individual human and animal sera, it was observed that both species and individual variations are great and that rabbit serum in general gave big differences in cultivation experiments with heated and non-heated sera. The present paper deals mainly with experiments with rabbit sera.

In tissue culture experiments and especially in cancer research interest seems to have been concentrated on looking for growth stimulating factors although the finding of possible growth controlling factors and of deficiencies in them might be of even greater importance. In some papers (1, 2) the »natural cytotoxins (heterotoxins)» in normal sera, which cause damage to heterologous cells, have been investigated. The authors of these papers also found that this effect could be removed by inactivation of the serum.

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<sup>1</sup> Aided by a grant from the Sigrid Jusélius Foundation.

Our finding that a similar phenomenon also occurs when human cancer cells (HeLa) are cultivated in human sera might indicate the existence of a growth controlling system in serum. In this connection the studies of Simms and Stillman (6) concerning the so-called B and C factors in chicken plasma and dog serum are interesting, as also the recent studies of the properdin system (5).

#### METHODS

The details of *the cultivation of the HeLa cells* and of the cell enumeration procedure have been published in an earlier paper (3). Briefly, the method consists of the cultivation of HeLa cells in 30 per cent human active serum with Hanks's solution in Roux bottles, the trypsinization of cells for inocula, the determination of inoculum variations, cultivation experiments in test tubes in triplicates or quadruples, and the final enumeration of the nuclei by the citric acid method. The sera to be investigated were used as a 30 per cent dilution in Hanks's solution, and the cultivation time was two days. The sera were investigated if possible the following day after collecting the blood.

The *absorption experiments* with HeLa cells and Zymosan was carried out as follows: HeLa cells cultivated in Roux bottles in 30 per cent human active serum were trypsinized, washed three times with Hanks's solution and counted. To six ml of active serum were added c.  $10^7$  HeLa cells. The suspension was incubated for 30 minutes in a 37° C waterbath with frequent, slight agitations. The cells used for absorption were then centrifuged at room temperature for 10 minutes at 1500 r.p.m. in an International Refrigerated Centrifuge using head No. 269. No cells were observed in the absorbed serum when studied in a counting chamber. Similarly treated serum without the addition and centrifugation of the HeLa cells was used as a control. The zymosan (Fleischmann Laboratories, U. S. A.) was boiled in distilled water for one hour before the serum was treated. Five mgm. of zymosan were then added for each ml. of serum. Otherwise the treatment was the same as with the HeLa cells.

#### RESULTS

##### *Inactivation Experiments*

*Effect of Different Temperatures.* — With an inactivation time of 30 minutes for the serum it was found that the lowest tempera-

ture at which some increase occurred in the cell counts as compared with the nonheated serum was at about 51° C. The increase became still greater up to 54°—56°C after with it began to fall off. Fig. 1 shows the results of some experiments.

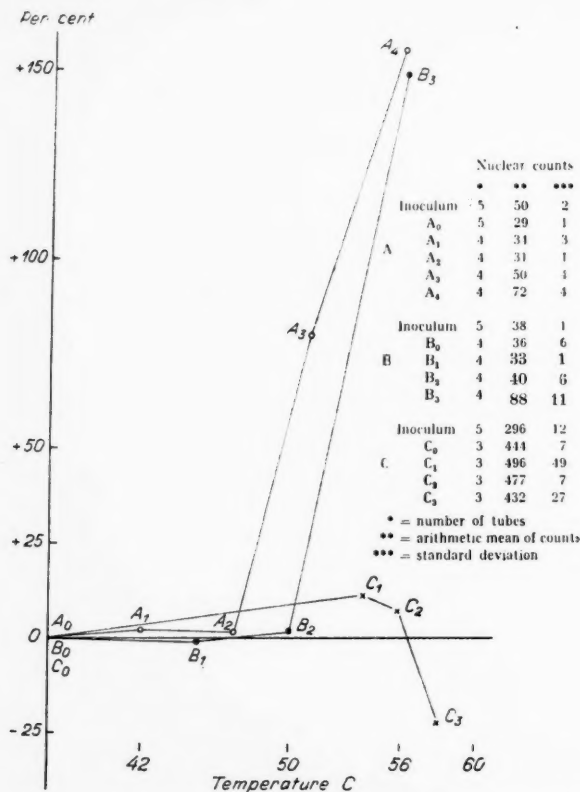


Fig. 1. — Percentual increase or decrease in two day counts of HeLa cells cultivated in rabbit serum heated for 30 minutes at different temperatures as compared with counts in nonheated serum. The inoculum size in thousands was A = 50, B = 38, C = 296.

After two days incubation the cell counts in nonheated sera may be even smaller than the inoculum, if small enough inocula are used. In this connection it has to be stressed that in experiments like these the inoculum size (and quality) is of the utmost importance. When large inocula are used none or very small differences are observed in the cell counts.

*Effect of Addition of Nonheated Serum to Inactivated Serum.* —

When small inocula were used the addition of only a small amount (three per cent of the total volume) of active serum to inactivated serum could be enough to reverse the effect of the inactivation (Fig. 2). When greater inocula were used more nonheated serum was needed to produce the same effect; a small amount of non-heated serum added to inactivated serum seems even to augment the growth of the cells.

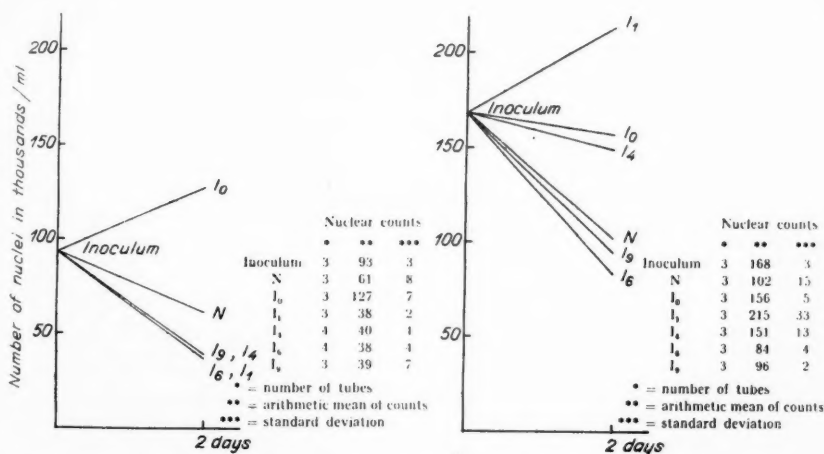


Fig. 2. — Growth of HeLa cells in normal rabbit serum (N), in serum inactivated by heating at 56°C for 30 minutes (I<sub>0</sub>), and in inactivated sera to which different amounts of active serum have been added (10% = I<sub>1</sub>, 40% = I<sub>4</sub>, 60% = I<sub>6</sub> and 90% = I<sub>9</sub>). Two experiments with different inoculum sizes.

*Absorption Experiments*

The inactivation experiments suggested the presence of heat labile factors controlling the growth of the HeLa cells. The great differences in the experiments with different inoculum sizes also suggested that only a part of the cells used as inocula can be destroyed or inhibited in their growth by the assumed growth controlling factors in the serum quantity in question. This led to the absorption experiments with HeLa cells.

Taking into consideration the possibility of the properdin-complement system as a growth controlling factor the experiments with zymosan, which is known to inactivate it, were carried out.



As can be seen from Fig. 3 which shows the results of one typical experiment, both HeLa cell absorption and the zymosan treatment of active serum have the same type of effect as inactivation.

The percentual increase in the counts as compared with serum heated to 37° C was after inactivation 71, after absorption with HeLa cells 92, after absorption with HeLa cells followed by inactivation 100, after zymosan treatment 100 and after zymosan

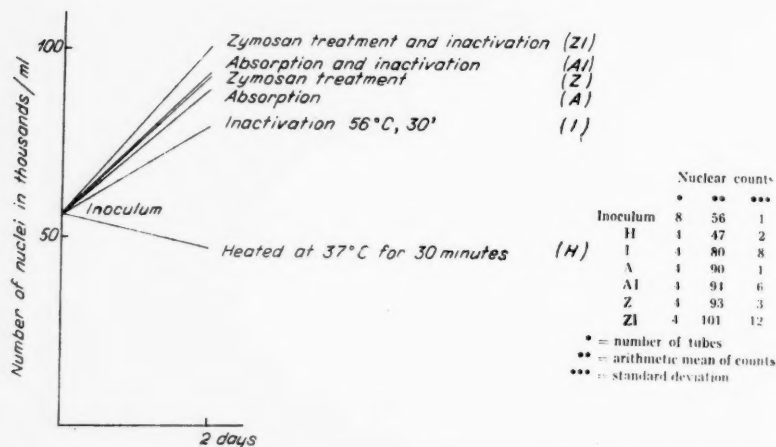


Fig. 3. — Growth of HeLa cells in serum heated at 37° C for 30 minutes (H), in serum inactivated by heating at 56° C for 30 minutes (I), in zymosan-treated serum (Z), in zymosan-treated serum followed by inactivation (ZI), in serum absorbed with HeLa cells (A) and in serum absorbed with HeLa cells and thereafter inactivated (AI).

treatment and inactivation 115. The differences between the effect of the control serum heated to 37° C and the effects of the absorbed sera are statistically highly significant.

#### DISCUSSION

The present experiments tend to indicate that thermolabile growth controlling factors exist in serum and that these can be adsorbed to HeLa cells and zymosan. The possibility that the HeLa cells condition the serum during the absorption time by

adding to it some metabolic products is less plausible, since the same result was observed after the zymosan treatment. The finding, published in our earlier paper (4) and now under extensive investigation, that human sera as well can be changed by inactivation does not make the assumption of »heterotoxins» active in rabbit serum necessary. Moreover, the finding reported by Miller and Hsu (2) that not all rabbit sera contain this heat labile growth inhibiting factor makes the assumption of a species specific heterotoxin questionable.

The possibility, however, that certain factors in human and rabbit serum act only against malignant cells and that it is not a question of a general growth controlling system must be taken into consideration, since only HeLa cells have so far been studied.

#### SUMMARY

The effect of rabbit serum on the growth of HeLa cells has been studied.

Heating of the serum at 56° C for 30 minutes increased cell counts in cultures of HeLa cells as compared with counts obtained in nonheated serum. The effect of the heating began at about 51° C.

Absorption of the serum with HeLa cells and treatment with zymosan both gave similar results to inactivation.

The great importance of the inoculum size and quality in studies like these is stressed.

The possible existence of a growth controlling system in serum is discussed.

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## SOME NEW BLOOD GROUP SPECIFIC PHYTAGGLUTININS

### A PRELIMINARY REPORT

by

O. MÄKELÄ and PIRJO MÄKELÄ

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Since 1948 when Renkonen (2) discovered the first plant agglutinins selectively agglutinating human red cells, much work has been done on this subject. An extensive monograph on phytagglutinins was published only recently, in which Krüpe (1) has compiled the most important findings so far, quite a few of them his own.

Frequent attempts have been made to substitute plant agglutinins for human test sera in routine ABO grouping. One of the reasons why failure has almost always been met with here is the absence of a good anti-B phytagglutinin. At present plant agglutinins probably find their primary use for routine work in A<sub>1</sub>A<sub>2</sub> subgrouping, secretor determination and possibly in MN grouping.

Working on the family *Leguminosae* in search for plant agglutinins (ca. 1000 species) we came upon a new anti-A<sub>1</sub> agglutinin in *Amphicarpaea bracteata* and *Amphicarpaea trisperma* of the tribe *Phaseolae*. In *Bandeiraea simplicifolia* of the tribe *Bauhiniae* we found what we believe is the purest known anti-B phytagglutinin. In all such *Virgilia* species as we studied, viz. *V. divaricata*, *V. lutea* and *V. oroboides*, a new agglutinin of the anti-H type was found. *Virgilia* belongs to the tribe *Sophorae*. Table 1 shows the titres of the crude extracts that were prepared according to the Renkonen technique from some of the above plants.

TABLE 1

TITRES OF THE CRUDE EXTRACTS AGAINST DIFFERENT KINDS OF RED CELLS

	Medium	Red Cells Tested					
		A <sub>1</sub>	A <sub>2</sub>	A <sub>1</sub> B	A <sub>2</sub> B	B	O
<i>Amphicarpaea bracteata</i>	NaCl	8	—	4	—	—	—
	AB-serum	64	16	64	—	—	—
<i>Bandeiraea simplicifolia</i>	NaCl	—	—	4	32	64	—
	AB-serum	—	—	16	64	128	—
<i>Virgilia lutea</i>	NaCl	—	1	—	—	1	2
	AB-serum	—	2	—	1	1	4

Recently harvested *Bandeiraea simplicifolia* seeds contained an agglutinin that reacted also with A-cells though less strongly. With two extracts prepared from the same seed sample a year later we tested 30 A<sub>1</sub>, 4 A<sub>2</sub> and 23 O blood specimens. The results were totally negative. In the meantime the appearance of the seeds had changed, they had become wrinkled and the extract made from them was an intense brown.

In the seeds of a few *Bauhinia* species, e.g. *Bauhinia purpurea*, we found an agglutinin with an unmistakable affinity to the agglutininogen N. It does not react in NaCl milieu. With it we tested 135 blood specimens; the results are shown in Table 2.

TABLE 2

SHOWING THE MN DISTRIBUTION OF THE BLOOD SPECIMENS TESTED WITH THE EXTRACT OF *Bauhinia purpurea* AND THE NUMBERS OF SPECIMENS WHICH ARE AGGLUTINATED BY THE EXTRACT IN DIFFERENT FINAL DILUTIONS

MN-group of the Cells	Numbers of Cell Specimens Agglutinated by Extract in Final Dilution of					
	No aggl.	1/1	1/2	1/4	1/8	1/16
M .....	41	1	1	—	—	—
MN .....	6	11	11	22	6	1
N .....	—	—	4	14	10	7
Totally	47	12	16	34	16	8

The Ss-groups of the blood specimens used in the study of the *Bauhinia* extracts were not determined. The agglutinin, however, is not likely to be an anti-s, as the frequency in Finnish population

of Ms chromosome is unusually high, or *ca.* 0.4. The considerable excess of N gene in the series is due to many of the examined persons being related to one another.

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## EFFECT OF BACITRACIN AND NEOMYCIN ON THE BACTERIAL FLORA OF THE COLON

by

O. PERÄSALO AND MIKKO PASILA

(Received for publication September 27, 1956)

In surgical measures carried out in the region of the alimentary canal, an infection originating from the intestinal lumen is among those which give rise to the severest complications. It is therefore natural that the effect of all available drugs from the first sulfa drugs to the most recent antibiotics should have been tried out on the bacterial flora of the intestine also. To serve its purpose here, a drug must destroy bacteria rapidly and effectively, it should be as slightly resorptive as possible, it should not cause irritation of the intestine nor form resistant strains. Among the drugs mainly used at the present day, sulfa drugs are especially effective on coliform bacteria. Their effect is usually rather slow and necessitates a long period of treatment. Streptomycin again affects an extensive spectrum, but it soon produces resistant strains. Tetracyclines are at present considered the best as far as coliform and anerobe bacteria are concerned (1). Yet even they give rise to certain complications. Malaise may result, diarrhoea and even quite severe vomiting. Sometimes, by combining various substances, eventual symptoms may be ameliorated and, what is more important, the effect of the drugs may be enhanced.

Neomycin and bacitracin are among the drugs which have recently come to the fore in this sense. These drugs have been known for a considerable time and have been used in various ways in local treatment. Being nonresorptive they suit this purpose

particularly well. Neomycin has an extensive antibiotic spectrum; it affects all aerobic bacteria but has a surprisingly slight effect on streptococcus-pneumococcus groups (5). The spectrum of bacitracin roughly corresponds to that of penicillin, i.e. it affects gr+ cocci and stabs. A combination of these substances shows a marked increase in efficiency. The two drugs, when combined, have also been found to become resorbed to a minimal extent only, 0.6—0.8% of the whole amount administered. Their bactericide effect is very rapid and no toxic intestinal or other symptoms have occurred (3). Opinions as to dosage are contradictory (4). According to the very latest research, an individual weighing 60 kg should be given 3 + 3 g a day of neomycin + bacitracin on three successive days at least. The primary purpose of the following study is to get an insight into the effect of the neomycin + bacitracin combination which we used, on the bacterial flora of the intestine.

#### MATERIAL AND METHOD

To elucidate the properties of the combination neomycin + bacitracin<sup>1</sup> a comparison with some drug prevalent in clinical treatment was found advisable. Phtalylsulfathiazole («Ftalazol»<sup>2</sup>) was chosen for this purpose.

The experimental studies were made on guinea pigs, comparing the effect of the bacitracin-neomycin combination with that of phtalylsulfathiazole on the bacteria of the guinea pig's colon. Fifteen guinea pigs were used for the former and thirteen for the latter experiments.

The average weight of the guinea pigs was 600 g. The control series comprised 4 guinea pigs. The amount of drug given to the test animals was 50 mg of bacitracin + neomycin and 70 mg of phtalylsulfathiazole each day on three days. The drugs were suspended into water and were given to the guinea pigs through a tube. After this they got no food, only water. After three days specimens were taken from the colon and the rectum. They were cultivated and differentiated by smearing some suspension

<sup>1</sup> The combination of bacitracin and neomycin used was preparation T 605 of «Orion», Manufacturing Chemists. The tablets contain 15,000 I.U. of bacitracin and 0.3 g of neomycin.

<sup>2</sup> «Ftalazol», used as a control medium, is a sulfa preparation made by «Orion», each tablet containing 0.5 g of phtalylsulfathiazole.



on a blood plate and on a Drigalsky plate<sup>1</sup>. The relative number of colonies was assessed. It is worth mentioning that the figures were given by the laboratory personnel blindly; they did not know in advance where the specimens came from.

The same examination was carried out on the human being. Six patients were given a total amount of 6 g per day on three days and three 8 g phtalylsulfathiazole per day likewise on three days. Because of the condition of the patients no surgery was indicated, and the specimens were therefore obtained with the rectoscope from a spot 30 cm from the anus with a cotton wad soaked in common salt. The specimens were cultivated and differentiated like those taken from the experimental animals.

### RESULTS

The examinations performed on the laboratory animals show that in the first part of the colon the initial values were comparatively high. After intake of bacitracin + neomycin the bacterial amount as far as gr + cocci and gr—stabs were concerned had fallen to about one third (Fig. 1). No noteworthy decline occurred for yeast and mould. The specimens obtained from the lower part of the rectum revealed still higher initial values, but the amounts of gr + cocci and gr—stabs and yeast and mould had decreased to almost zero (Fig. 3). In the determinations made with phtalylsulfathiazole in the first part of the colon, no appreciable fall took place as far as the cocci were concerned, but a major disappearance, instead, of gr—stabs and yeast and mould (Fig. 2). In the rectum the amount of gr—stabs and gr + cocci decreased to about half of the original (Fig. 4.)

In the determinations carried out on man in the region of the rectum, an almost total disappearance of *escherichia coli*, *micrococcus pyogenis* and *pseudomonas aeruginosa* was noted, while no appreciable decrease occurred in the yeast fungus, with neomycin and bacitracin (Fig. 5). Phtalylsulfathiazole produced (Fig. 6) a fall of *micrococcus pyogenes* down to about a half, of *escherichia coli* and *pseudomonas aeruginosa* to about a quarter and of the yeast fungus to a third of their initial amounts.

<sup>1</sup> The laboratory examinations were carried out in the Microbiological Department of the Manufacturing Chemists «Orion».

Fig. 1. — The effect of the bacitracin+neomycin combination on the bacterial flora of the first part of the colon in 15 guinea pigs.

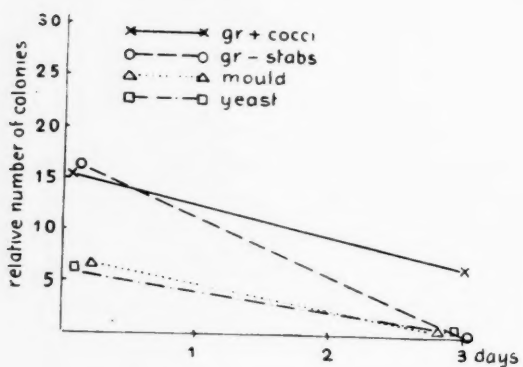
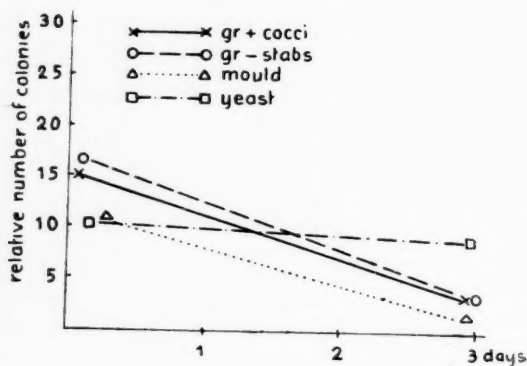
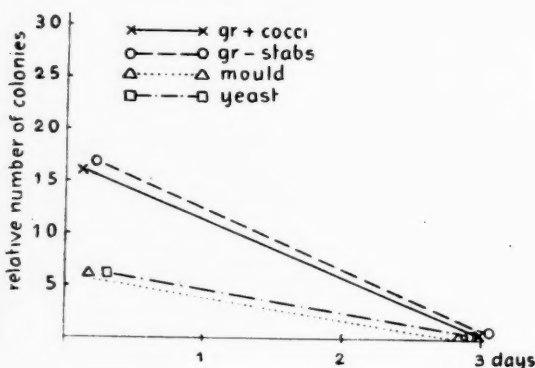


Fig. 2. — The effect of the sulfa preparation on the bacterial flora of the first part of the colon in 13 guinea pigs.

Fig. 3. — The effect of the bacitracin + neomycin combination on the end part of the colon in 15 guinea pigs.



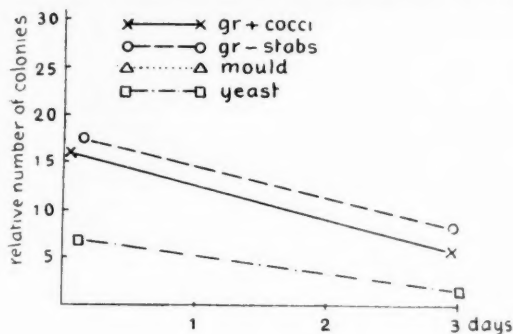


Fig. 4. — The effect of the sulfa preparation on the bacterial flora of the end part of the colon in 13 guinea pigs.

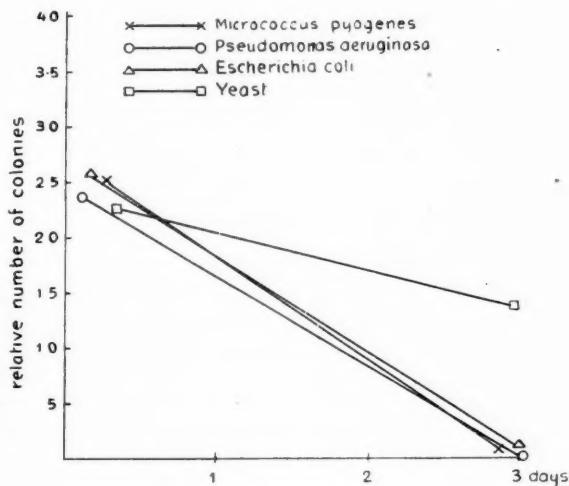


Fig. 5. — The effect of bacitracin + neomycin on the bacterial flora of the rectum in the human being.

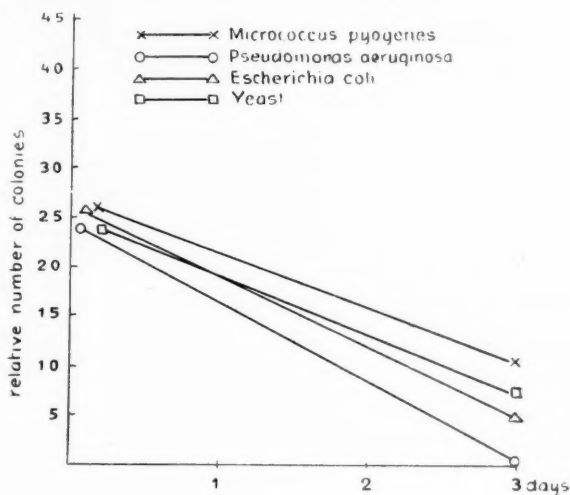


Fig. 6. — The effect of the sulfa preparation on the bacterial flora in the rectum of the human being.

## COMMENT

An assessment of the results obtained shows a clearly enhanced effect from the use of the bacitracin + neomycin combination on the bacterial flora of the colon. According to the examinations, this substance is also more effective than phthalylsulfathiazole, the control medium. It seemed from the experimental series that the sulfa preparation used had a somewhat greater sterilizing capacity in the first part of the colon than bacitracin + neomycin. Yet in the end part of the colon bacitracin + neomycin was preponderantly more effective. This can probably be explained as due mainly to the slighter resorption of the combination neomycin + bacitracin compared with the sulfa preparation. The conclusions that can be drawn from the series of experiments carried out of course remain open to qualifications. In the first place, the bacterial flora of laboratory animals varies considerably, and also differs from that in man. Yet in the present investigation the findings of the effect of the drugs on laboratory animals and man tally to a very great extent, so that apparently the bacitracin + neomycin combination is more effective especially on the bacterial flora of the end part of the colon than sulfa preparations. Results with the same trend have been obtained for instance by Herzberg and Loe (2).

## SUMMARY

The effect of the combination bacitracin + neomycin on bacterial flora of the colon was studied using a sulfa preparation as a control medium. The examinations were carried out on laboratory animals and man. The bacitracin + neomycin combination was found to be clearly more effective than phthalylsulfathiazole, especially on the bacterial flora of the end part of the colon.

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## INFLUENCE OF HYPERTHYROIDISM AND HYPOTHYROIDISM ON GUINEA-PIG TUBERCULOSIS

by

OLE WASZ-HÖCKERT, ALF BACKMAN and HÅKAN POPPIUS

(Received for publication October 1956)

The present investigation is one of a series of host-parasite relationship studies (7, 8, 9) made by us. Its purpose was to find out whether changes in the metabolism governed by the thyroid gland affect the course of the infection in tuberculosis. We employed thyroidin to induce hyperthyroidism in the experimental animals, and the thyrostatics, methylthiouracil and sulfathiourea, which block the thyroidal function, to induce hypothyroidism.

The investigation was divided into two experiments; in the first we infected animals, already treated, while in the second the treatment was begun simultaneously with the tuberculosis inoculation.

### EXPERIMENT I

*Material.* — 40 guinea-pigs, mean weight 500 g, were used for the experiment. The animals were tested with 1.0 mg of Old-Tuberculin and found to give a negative tuberculin reaction. The animals were divided into four groups:

Group A: 10 animals, control group, no treatment.

Group B: 10 animals, treated with 0.05 g of powdered thyroidin<sup>1</sup> per day in a water suspension, with a hose, through the mouth.

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Aided by a grant from the Sigrid Jusélius Foundation.

<sup>1</sup> Thyreoidea. »Medica»

Group C: 10 animals, treated with 0.05 g of sulfathiourea<sup>2</sup> per day, administered as in Group B.

Group D: 10 animals, treated with 0.05 g of methylthiouracil<sup>3</sup> per day, administered as in Group B.

30 days after the treatment was begun the animals were inoculated in the right inguinal fold with 0.2 cc of a 7-day culture of *M. tuberculosis* H<sub>37</sub>Rv, diluted to 10<sup>-1</sup>. The treatment with thyroïdin, sulfathiourea and methylthiouracil continued for another 30 days, after which the animals were sacrificed.

*Results.* — 1. The autopsy was made blindly, i.e. the performer of the autopsy did not know to which group an animal belonged. The macroscopic tuberculous lesions at the point of inoculation, in the regional lymph nodes, lungs, liver and spleen were examined. The findings are illustrated diagrammatically in the Fig. 1.

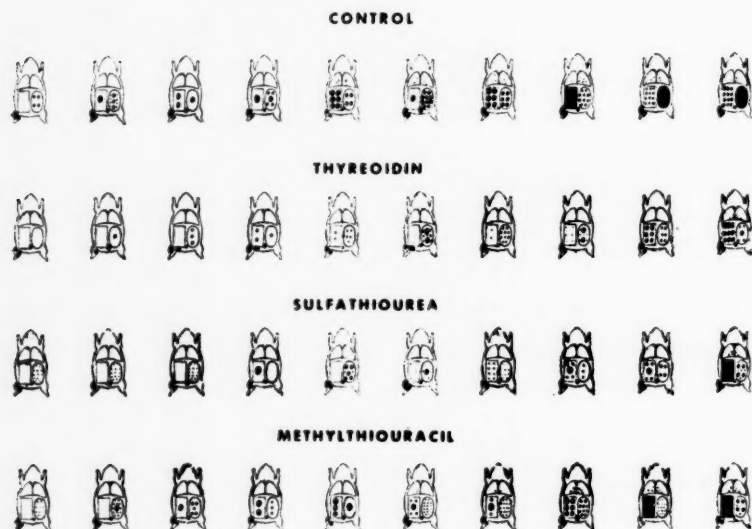


Fig. 1.

After five weeks, the number of guinea-pigs with more serious tuberculous changes was greater in the control and methylthiouracil groups than in the others.. This is also given numerically in

<sup>2</sup> Badional, «Bayer»

<sup>3</sup> Methylthiouracil, «Medica»

Table 1, which gives the mean values of the tuberculous lesions per organ and group and their total computed according to Feldman's principle (3, 4).

TABLE 1

	Spleen	Lungs	Liver	Inoculation	Total
Group A .....	21	6	15	10	52
» B.....	13	1	9	10	33
» C.....	13	1	11	10	35
» D.....	17	5	15	10	47

2. During the course of the experiment the animals were weighed weekly. The weights are given in Fig. 2.

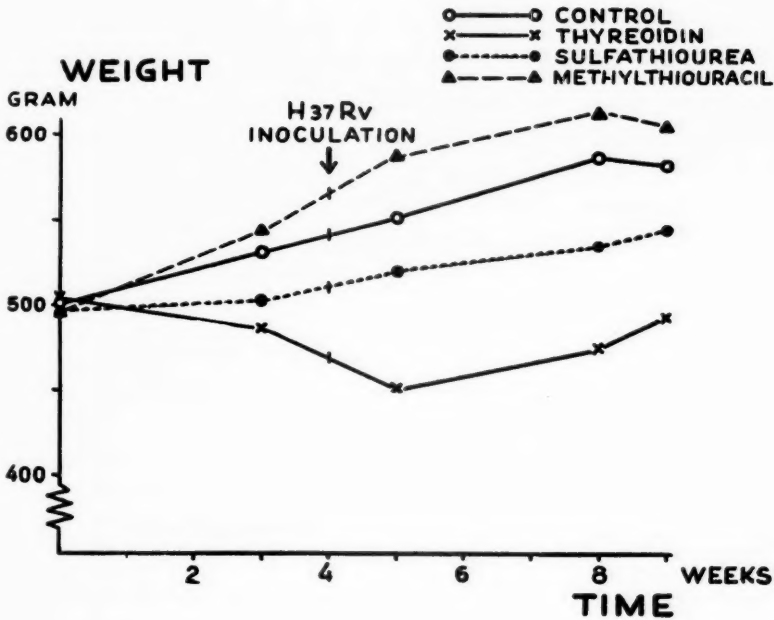


Fig. 2.

The animals treated with thyroïdin showed definite signs of thyrotoxicosis by the time of autopsy, viz. loss of weight, dehydration and loss of hair.



## EXPERIMENT II

*Material.* — 72 guinea-pigs, mean weight 400 g, divided into two main groups.

Group 1: 56 animals were inoculated in the right inguinal fold with 0.2 cc of a 7-day culture of *M. tuberculosis*, H<sub>37</sub>Rv, diluted to 10<sup>-1</sup>. These animals were divided into 4 groups:

- A. 14 animals, control group, no treatment.
- B. 14 animals, treated with 0.05 g of powdered thyroïdin per day in a water suspension, with a hose, through the mouth.
- C. 14 animals, treated with 0.05 g of sulfathiourea per day, administered as in Group B.
- D. 14 animals, treated with 0.05 g of methylthiouracil per day, administered as in Group B.

Group 2: 16 animals were not inoculated. Like the inoculated animals, they were divided into 4 groups.

- A. 4 animals, control group, no treatment.
- B. 4 animals, treated with 0.05 g of powdered thyroïdin per day, administered as in Group 1 B.
- C. 4 animals, treated with 0.05 g of sulfathiourea per day, administered as in Group 1 B.
- D. 4 animals, treated with 0.05 g of methylthiouracil per day, administered as in Group 1 B.

The inoculated animals were autopsied 4, 10, 12 and 15 weeks after challenge and the introduction of treatment, the uninoculated 15 weeks after treatment was started.

It must be pointed out that the last 5 guinea-pigs in the thyroid group died a few days before the planned autopsy. That the animals were burned without our knowledge was an »laboratory accident». Hence, no full analysis can be made of the thyroid group. However, just before death the animals showed marked thyrotoxic symptoms, and we presume that their tuberculous changes were considerable. The guinea-pigs of the uninfected group were severely thyrotoxic at the same time, but none of them (4 animals) died.

*Results.* — 1. The results, illustrated in Fig. 3, show that the tuberculous infection in Experiment II took a milder course than in Experiment I. One of the causative factors is probably that we

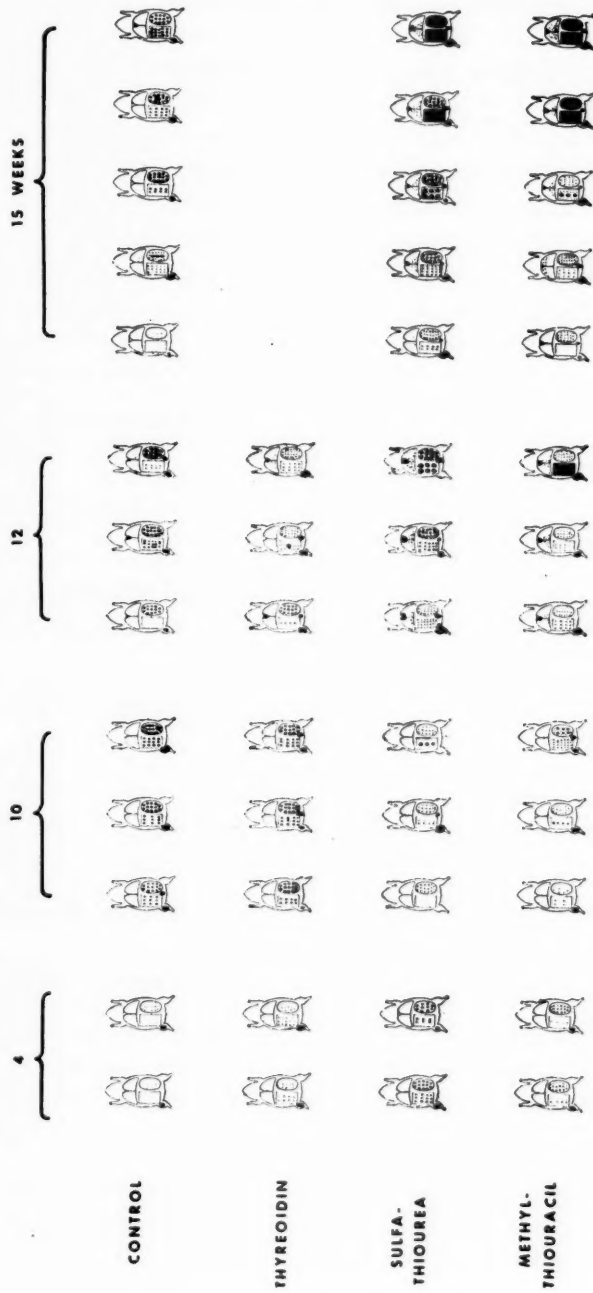


Fig. 3.

employed in these two experiment two different  $H_{37}Rv$  strains (imported 1953 and 1956, respectively), because we wanted a milder infection in the later series (Experiment II).

By the end of 10 weeks no difference was discernible between the groups, but at 12 and 15 weeks the tuberculous changes were aggravated. As mentioned above, no 15-week results are available for the thyroid group, but 12 weeks after the inoculation the results complied well with those obtained in Experiment I.

2. The same is noted when the results are computed according to Feldman's principle (Table 2).

TABLE 2

	4	10	12	15 Weeks
Control .....	30	43	43	43
Thyreoidin .....	30	46	43	—
Sulfathiourea .....	36	24	65	67
Methylthiouracil .....	30	33	48	62

3. In the course of the experiment the animals were weighed weekly. The mean weights for Group 1, the TB inoculated animals, were:

	At the Beginning of the Experiment	At the End
Group A (control) .....	405 g	610 g
» B (thyroidin) .....	420 »	400 »
» C (sulfathiourea) ....	390 »	455 »
» D (methylthiouracil)..	410 »	575 »

The mean weights for Group 2, Experiment II, the uninoculated animals) were:

	At the Beginning of the Experiment	At the End
Group A (control) .....	420 g	500 g
» B (thyroidin) .....	415 »	340 »
» C (sulfathiourea) ....	415 »	480 »
» D (methylthiouracil)..	410 »	540 »

In a diagrammatic illustration of the weights a distinct trend line could be drawn for each group. As this tendency is clearly seen in the tables giving only the initial and final weights, the diagrammatic illustration can be omitted.

4. The adrenals of the animals were weighed on autopsy, the weight being calculated in mg per 100 g of the animal's weight. The results were computed as means per group and are given in Table 3.

TABLE 3  
WEIGHT OF THE ADRENALS IN MG AND MG % OF THE ANIMAL'S WEIGHT

		10 Weeks		12 Weeks		15 Weeks	
		mg	mg %	mg	mg %	mg	mg %
Experiment II, Group 1, TB- inoculated animals	Group A (control)	270	52	296	67	405	68
	Group B (thyroidin)	370	60	457	78	—	—
	Group C (sulfathiourea)	270	59	333	62	335	71
	Group D (methylthiour- acil)	270	52	323	59	373	65
Experiment II, Group 2, unin- oculated animals	Group A (control)					220	44
	Group B (thyroidin)					397	113
	Group C (sulfathiourea)					347	76
	Group D (methylthiour- acil)					345	63

In Experiment II, the weight of the adrenals was checked on autopsy. This was done to make sure that the differences between the tuberculous changes in the various groups were not due to the stress effect of hose feeding. This check was therefore carried out both for the series of guinea-pigs not infected with tuberculosis and for the tuberculous series. It turned out that hose feeding alone may to a certain extent have affected the adrenal weight of the normal group. Thyroidin also seems to have had a distinct effect on the adrenal weight, a finding compatible with the studies effected on rats (1).

## DISCUSSION

Our purpose was to affect the resistance of the host organism to infection by a thyroid-governed change in the metabolism. Thyroidin, sulfathiourea and methylthiouracil were well suited for the purpose. However, it is known that thiourea and thiouracil derivatives have a tuberculostatic effect *in vitro* and *in vivo* (2).

The relationship between thyroid function and tuberculosis, both clinically and experimentally, has been referred by Schäfer (6).

As early as 1921, Webb (10) showed that thyroidectomized guinea-pigs showed severer forms of tuberculosis than control animals.

The data presented in the later literature, however, is controversial regarding the effect of both thyroidectomy and thyroxin treatment on guinea-pig tuberculosis. Our results indicate that hypothyroidism produced by sulfathiourea and methylthiouracil lowers the resistance and hence the tuberculous infection takes a more severe course. But hyperthyroidism, produced by thyroid medication, had a favourable effect on the tuberculous infection. In severe thyrotoxicosis, however, the resistance of the host seemed to be weakened and the tuberculous processes were more extensive. We believe that this was shown in two forms of tuberculosis, one more acute (30 days) induced by a stronger  $H_{37}Rv$ -inoculum, and the other of a more chronic type (105 days) induced by a weaker inoculum.

This reduction, therefore, shows that thyroid function also in so far as we were able to affect it experimentally, participates in the resistance mechanism against infections, in this case chronic, tuberculous infection. How this takes place we cannot say, but our observations of the adrenal weights seem to suggest that the influence derives from the thyroid via the pituitary gland to the adrenals, a view advanced by Kracht (5).

## SUMMARY

The authors made an experimental study of 112 guinea-pigs in order to investigate the influence of thyroid function on the course of tuberculous infection. Hypothyroidism was produced by sulfathiourea and methylthiouracil medication, hyperthyroidism by the administration of thyroidin.

Hypothyroidism aggravated and hyperthyroidism eased the course of the disease to some extent.

The weight of the adrenals was investigated and the adrenocortical function is discussed.

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## EXPERIMENTAL FACTORS INFLUENCING ANAPHYLACTIC SHOCK IN GUINEA-PIGS

PRELIMINARY REPORT

by

OLE WASZ-HÖCKERT and MARTTI KOKKONEN

(Received for publication October 11, 1956)

With the intention of later studying factors influencing allergy we have started a series of experimental studies with the use of anaphylactic shock on animals. In this first study we observed how the blood volume and movements of the animals affect the anaphylactic shock. The guinea-pig, whose lungs constitute the shock organ, was selected as the experimental animal.

### MATERIAL AND METHODS

The study comprised 90 guinea-pigs whose weights ranged from 640—800 g.

*Anaphylactic Shock.* — The guinea-pigs were sensitized with 0.25 ml of active horse serum injected subcutaneously. The anaphylactic shock was produced 3—4 weeks later by injecting 2.5 ml of horse serum intracardially. The resulting shock symptoms were observed with a stopwatch and recorded. The symptoms were classified according to their intensity as functions of time, as follows:

1. Restlessness (the animal scratched its face, blinked its eyes and bit)
2. Sneezing, hairs began to bristle
3. Respiratory difficulties, coughing, retarded respiration.



4. Convulsions (spasms of the muscles of the neck, abdomen and back)
5. The animal was unable to stand on its feet, panted for breath
6. The animal lay on its side kicking. Respiration paralysed. Last convulsions.
7. Inertness. Diaphragm paralysed.
8. Death.

*Measuring the Blood Volume.* — The blood volumes were determined by the Evans blue method.

*Lung Volume on Autopsy.* — The lung volume was determined by a pneumometer. The guinea-pigs were autopsied after the respiratory centre had stopped functioning. The trachea was ligated cranially of the bifurcation and the emphysematous lungs were taken out after the heart had been removed. The volume was determined by immersing the lungs on the tip of a glass rod into a graded measuring flask. The volume of the glass rod and binding material was deducted from the reading obtained. The volumes varied from 16—28 cc.

#### TECHNIQUE OF THE EXPERIMENTS

*Experiment I.* — 24 guinea-pigs (640—780 g) were sensitized and then shocked 3 weeks later; they were divided into the following groups according to the way in which they spent the interval:

- A. Control group, which moved freely. 6 guinea-pigs had a moving space of 1.2 sq m.
- B. Inactive group, 6 guinea-pigs. Each animal was kept separately in a special box where it was unable to move; it had sufficient food and water within reach.
- C. Training group, which moved freely, like Group A. In addition, each guinea-pig ran an average of 1.4 km in a training device during one hour a day for 3 weeks. The diameter of the training device running wheel was 50 cm and it revolved at 18—20 RPM.
- D. Bled group. Each guinea-pig was bled every week of 7.5 cc of blood by intracardial injection needle. The animals were otherwise allowed to move freely, like Groups A and C.

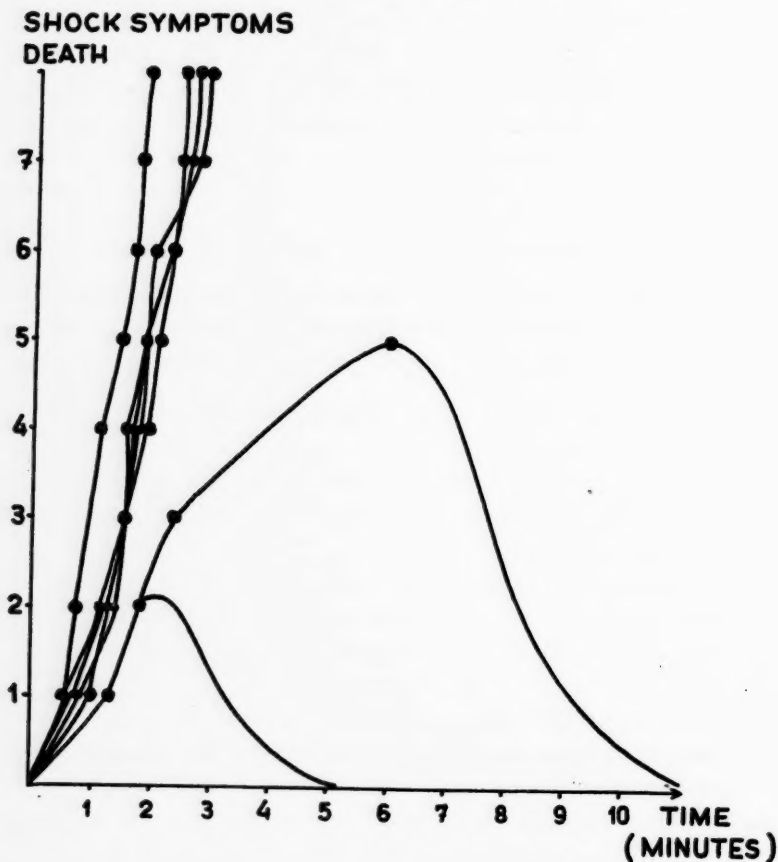
In addition, the blood volume and haematocrit of 6 normal guinea-pigs was determined.

*Experiment II.* — 60 guinea-pigs (550—800 g, average 605 g) were divided into two groups. They were sensitized, and shocked four weeks later with active horse serum. During the period of sensitization their classification was:

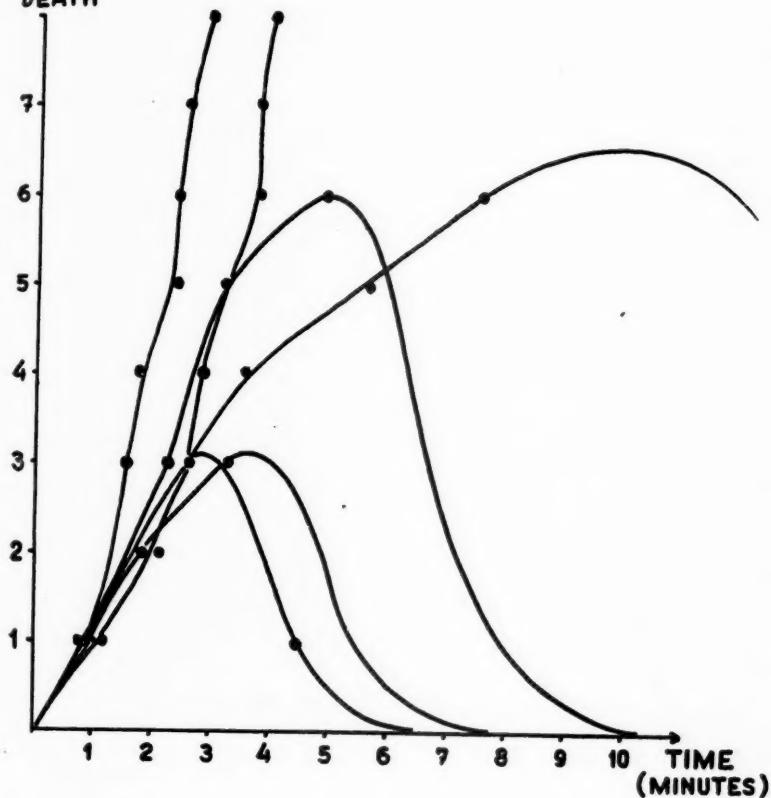
- A. Control group, 30 guinea-pigs, which were allowed to move freely: 1.2 sq m of moving space per 15 guinea-pigs.
- B. Inactive group. Moving space 0.2 sq m per 15 guinea-pigs. The guinea-pigs were kept in relative inactivity and fed ad libitum.

#### RESULTS

*Experiment I.* — The anaphylactic shock symptoms in the various experimental groups are illustrated in Figs. 1—4.



Figs. 1—4. — Distribution of the shock symptoms as measured in minutes. The different graphs represent: control (Fig. 1), inactive (Fig. 2), training (Fig. 3) and bled (Fig. 4) groups respectively.

SHOCK SYMPTOMS  
DEATH

Figs. 2

The shock symptoms in the control group (Fig. 1) and the training group (Fig. 3) were highly similar in intensity. In each group, two guinea-pigs died of the shock and two recovered! In the remaining groups, inactive and bled (Figs. 2 and 4), the shock symptoms were milder. The blood volume and haematocrit of the respective groups were:

The results are presented as mean values as no significant difference was demonstrable, apart from the bled guinea-pigs which showed lower haematocrit values.

*Experiment II.* — As we intend with a larger animal material to study whether a significant difference is obtainable, we con-

TABLE 1  
MEAN BLOOD VOLUME AND HAEMATOCRIT VALUES

	Blood Volume % by Weight	Haematocrit
Normal .....	7.1	33.2
Control .....	9.9	41.2
Inactive .....	7.8	37.8
Training .....	10.7	40.7
Bled.....	9.4	26.5

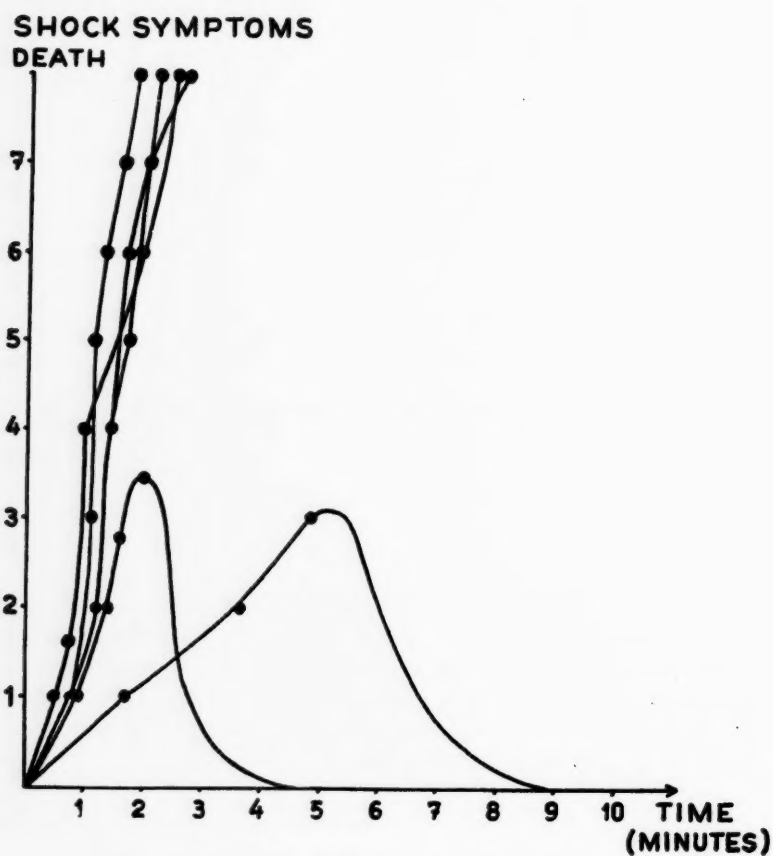


Fig. 3

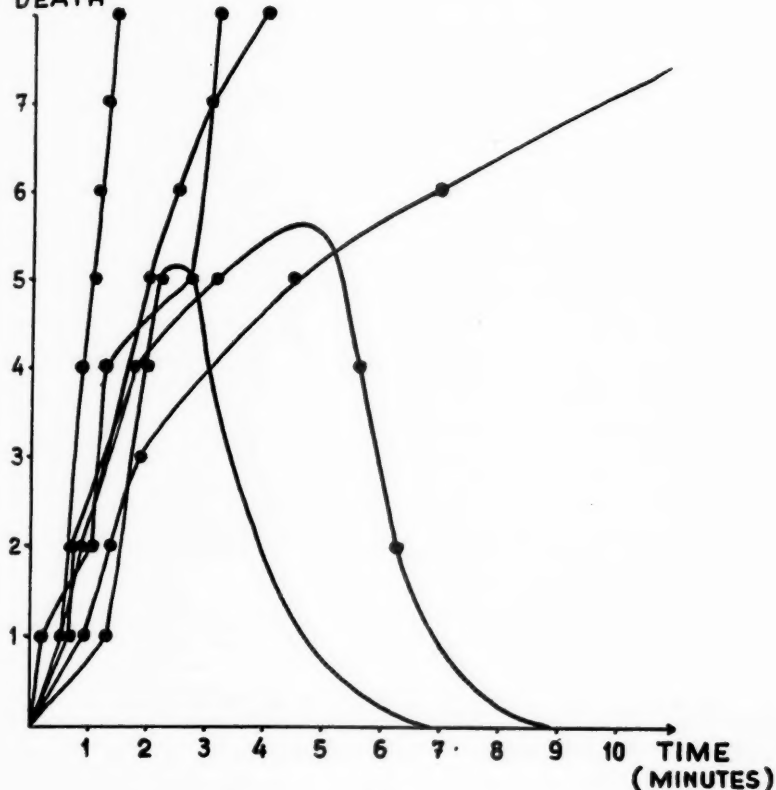
SHOCK SYMPTOMS  
DEATH

Fig. 4

centrated here on only two groups, the control and the inactive groups.

We have reported the females separately as during the 4-week period of sensitization in the crowded cage they generally lay

TABLE 2  
NUMBER OF RECOVERIES OR DEATHS IN ANAPHYLACTIC SHOCK.

Group	Recovered from Anaphylactic Shock Total (Female)		Death in Anaphylactic Shock Total (Female)	
Control .....	1	(1)	29	(14)
Inactive .....	5	(4)	25	(11)

down quietly, hence fulfilling the experimental purpose better than the males who suffered from the crowded conditions and fought.

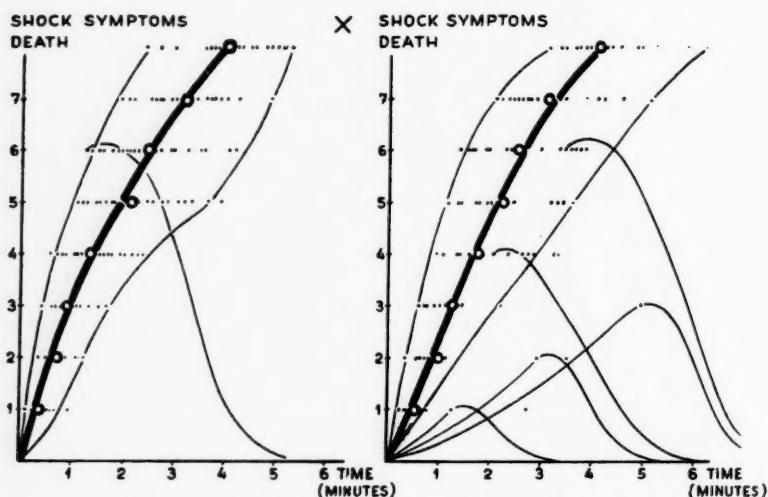


Fig. 5. — Distribution of the shock symptoms as measured in minutes. A thick trend curve has been plotted for the mean values.

There was thus no significant difference in this experiment as far as the shock symptoms were concerned, but fewer animals recovered in the control group. In this respect the female groups showed more of a difference, probably for the reasons mentioned above.

The lung volumes of the guinea-pigs that died of shock were 21.2 cc in the control group and 18.8 cc in the inactive group; the former represents a mean for 29 and the latter for 25 guinea-pigs.

#### DISCUSSION

In our first experiment the complete inactivity of the test animal during 3 weeks and a considerable weekly bleeding seemed to relieve the shock symptoms. However, when we reproduced the experiment we could not obtain significant differences. This may have been due in part to the other stresses affecting the male

animals. Fully aware of the large number of factors involved in a study of this kind, we make no attempt at drawing conclusions since conditions abnormal for the animal may influence the shock via the adrenals. Great differences have been noted in the size and histology of the adrenals of rats living free and those in captivity (1). We intend to make observations of the adrenal in our continuation studies. Similarly, the ability to form antibodies, etc., must be followed.

Räihä found in 1933 a right axis deviation of the electrical axis in the heart of asthmatic children. He later suggested that heart size and blood volume may be connected with allergy. A background for this idea is provided by the investigation into the cardiac volume of the mothers of premature babies (2).

Our experimental work does not, at this stage, provide any support for this hypothesis. We hope that our continued investigations, however, will shed additional light on the problem. The guinea-pig, whose shock organs are the lungs, is extremely well suited for these experiments.

#### SUMMARY

The authors studied the effect of activity, inactivity and bleeding on anaphylactic shock on guinea-pigs. Although differences were seen in a pilot test no significant differences were stated in a large series.

Since the report is of preliminary nature and the studies are to be taken further, no conclusions can be drawn.

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## THE EFFECT OF ZYMOBAN ON SUSCEPTIBILITY TO STAPHYLOCOCCAL INFECTIONS IN MICE

by

OLE WASZ-HÖCKERT, NILS OKER-BLOM and TIMO KOSUNEN

(Received for publication October 11, 1956)

The recent discovery and isolation by Pillemer and coworkers (2) of properdin, a serum protein, adds to the knowledge of the field of natural immunity. As Pillemer *et al.* (4) say: »Properdin in conjunction with  $Mg^{++}$  and serum cofactors resembling the components of complement kills or inactivates certain bacteria and viruses and participates in the lysis of abnormal red cells». High molecular weight polysaccharide complexes and the cell walls of many bacteria form a complex with properdin (4), thus inactivating the properdin system.

An insoluble carbohydrate complex derived from yeast cell walls, zymosan, inactivates the properdin-system in vivo and, furthermore, »intravenous injection of small doses of zymosan causes a rapid fall in the properdin titer within 1 to 2 hours, followed, after 2 to 14 days, by a marked rise in titer 200 to 300 per cent above the normal level» (3). Zymosan is thus evidently highly suitable for use in studies concerned with experimentally induced disturbances in the host's resistance to infections. This has been demonstrated for *E. coli* infections in mice and rats (5): »mice became highly susceptible to infection with an *E. coli* strain that was avirulent to normal mice when zymosan was injected intravenously, and the mice were resistant to a different *E. coli* strain that was virulent for normal mice» (3).

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Aided by a grant from the Sigrid Jusélius Foundation.

The significance of the properdin system in virus infections has also been discussed (2).

Experiments concerned with the effect of zymosan on the susceptibility of animals to different virus infections have therefore been initiated and will be reported on later (1).

The aim in the present investigation, however, has been to study the influence of zymosan on a virulent staphylococcal infection in mice. The criteria employed were the survival time, and the bacterial population measured quantitatively with the bacterial enumeration technique described previously (6).

#### MATERIAL AND METHODS

1. Mice. — Swiss Albino Webster male mice, ranging in weight 20—30 g, were used in the bacterial population study and for the adult group of the survival experiment. For the infantile group, however, albino mice, strain BLH, were used, ranging in weight 9—13 g.
2. Bacterial culture: — *Staphylococcus aureus*, strain «Orion», cultured for 12 hours in broth, was used in a 1:2 dilution. The challenge dose was 0.2 ml intravenously in one of the tail veins.
3. Zymosan: — 0.2 ml of a 10 mg/ml suspension was injected intravenously into a tail vein. The zymosan was obtained from The Fleischmann Laboratories, Standard Brands Incorporated, New York.
4. Bacterial enumeration technique: — The method described previously (5) was employed. In this experiment, however, the staphylococci were quantitatively determined from the liver and spleen also.

#### PLAN OF THE EXPERIMENT

76 mice were used for the experiments, challenged, divided into groups and treated as follows:

1. Survival. — 30 mice were injected intravenously with Zymosan. 1 hour later 20 of them were challenged with the staphylococci inoculum. One group of 16 mice was only inoculated

with staphylococci. The group and weights of the animals were thus as follows:

<i>The Infantile Group</i> .....	(9—13 g)
Zymosan .....	(5 mice)
Staphylococci .....	(8 » )
Zymosan plus staphylococci .....	(9 » )
<i>The Adult Group</i> .....	(20—30 g)
Zymosan .....	(5 mice)
Staphylococci .....	(8 » )
Zymosan plus staphylococci .....	(11 » )

The survival time was then determined.

2. Bacterial Enumeration. — The number of staphylococci in the organs — kidneys, liver and spleen — were determined each day as the mice were sacrificed. The values are given in logarithms.

## RESULTS

### 1. Survival.

Infantile Mice	Number of Mice	Cumulative Number of Deaths			
		2	4	6	8 Days
Zymosan .....	5	0	0	0	0
Staphylococci.....	8	0	1	1	1
Zymosan plus staphylococci ..	9	2	4	6	8

Infantile mice of BLH-stain were thus very susceptible to staphylococci in the zymosan treated group: 8 out of 9 mice died in comparison with practically nil in the other groups.

Adult Mice	Number of Mice	Cumulative Number of Deaths			
		2	4	6	8 Days
Zymosan .....	5	0	0	0	0
Staphylococci.....	8	0	0	0	0
Zymosan plus staphylococci ..	11	0	1	1	1

In the adult group, however, no difference could be stated.

2. *Bacterial Enumeration.* — The viable number of staphylococci in the organs are given in Table 1.

TABLE 1

Organs Investigated	Groups	Log. of Viable Number of Staphylococci							
		1.	2.	3.	4.	5.	6.	7.	11. Days
Kidney	Staphyloc.	6.14	6.36	8.55	8.20	8.42	8.30	7.93	7.98
		6.52	8.05	8.79	8.15	8.79	8.30		
	Staph. + Zym.	7.66	7.77	8.06	8.39	7.18	8.25	8.51	7.16
		7.10	7.92	7.12	7.02	7.53	7.24	6.68	7.63
Liver	Staphyloc.	5.24	3.95	3.76	3.51	0	0	0	0
		4.48	2.81	2.81	0	3.03	0	0	0
	Staph. + Zym.	4.71	5.71	4.28	0	2.95	0	0	0
		4.21	3.34	3.25	3.78	0	0	0	0
Spleen	Staphyloc.	5.08	3.71	2.65	3.63	0	0	0	0
		4.58	3.47	3.20	0	0	0	0	0
	Staph. + Zym.	4.80	4.62	4.29	0	2.89	0	0	0
		4.29	3.58	0	2.99	0	0	0	0

It will be seen that from the kidneys the number of staphylococci is about one log. more on the first and second day. But at the end of a week, on the contrary, the values are somewhat lower although not significantly. As for the liver and spleen, from the sixth day on no bacilli were detectable prior to which no difference could be seen between the two groups.

#### DISCUSSION

Our study seems to indicate that zymosan increases the susceptibility of young, infantile mice to staphylococcal infection, a result which emerged clearly from the survival experiment. This is true of the mice strain used and the staphylococci strain also. The same dose of bacterial inoculum given to another adult mice strain, however, showed no parallel reactions.

In the bacterial enumeration study a slight difference was noted on the first two days after challenge from the cultured kidney-tissue homogenice.

It seems obvious therefore that the inoculated staphylococci population multiplies very much at the same rate independently

of the single zymosan dose given. Taking the 1—2 day values into consideration, however, it might be worth while repeating the zymosan injections in further studies. In young mice of another breed an overwhelming infection caused death, the host-resistance having been weakened.

In these studies, of preliminary nature, properdin levels were not determined. Different mice strains will be used in further studies.

#### SUMMARY

Zymosan was injected intravenously into mice one hour before challenge with virulent staphylococci. The results were then studied as regards survival time and quantitative bacterial enumeration. In infant mice, the susceptibility to staphylococci was highly increased. No other significant differences were stated.

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## AN ATTEMPT AT SHORTENING THE DIAGNOSTIC GUINEA-PIG TEST

by

OLE WASZ-HÖCKERT and ALF BACKMAN

(Received for publication October 11, 1956)

As is known, the diagnostic guinea-pig test in tuberculosis takes six weeks. It would be of great value to the clinician if this period could be reduced. We have tried in the present study, by »stressing» the experimental animals with ACTH, to find out whether it is possible thus to shorten the guinea-pig test.

The study was divided into two experiments; the first was a pilot test to study the date at which results became visible, and the second was a large series to verify the results.

### EXPERIMENT I

Material: 16 guinea-pigs, mean weight 465 g, were used for the experiment. The animals were Mantoux tested (1.0 mg Alt-Tuberculin) and found to give a negative reaction. They were inoculated in the inguinal fold with 0.2 cc of a 7-day culture of *M. tuberculosis* H<sub>37</sub>Rv, diluted 10<sup>-1</sup>. The animals were divided into two groups:

Group A: control, no treatment.

Group B: the animals of this group were treated with ACTH<sup>1</sup>, 2 mg subcutaneously every two days. Two animals of each group were sacrificed and autopsied 3, 5, 6 and 7 weeks after the inoculation.

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Aided by a grant from the Sigrid Jusélius Foundation.

<sup>1</sup> ACTORION, Orion, Helsinki.

*Results.* — The results obtained were as follows:

1. Autopsy. Two animals of each group were sacrificed and autopsied 3, 5, 6 and 7 weeks after the inoculation. The autopsy was blind, i.e. the performer did not know the group to which the autopsied animal belonged. The site of inoculation, the regional lymph glands, liver, lungs and spleen were examined macroscopically. The result is shown diagrammatically in Fig. 1.

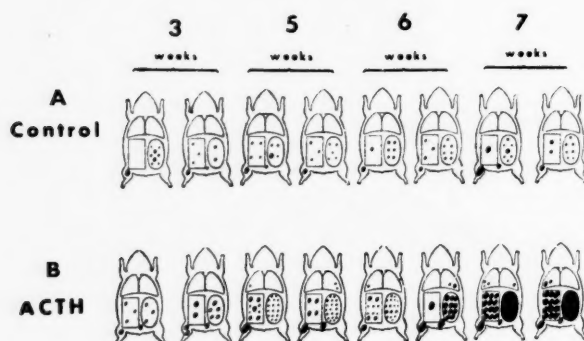


Fig. 1.

The figure shows that a distinct difference is discernible between the animals of the two groups at 5 weeks and later, but not at 3 weeks.

2. The results have also been computed according to Feldman's (2, 4) method and are given in Table 1.

TABLE 1

	3	5	6	7 weeks
Group A (control) . . . . .	25	30	30	30
Group B (ACTH) . . . . .	30	55	55	80

A distinct numerical difference between the groups is observable as from 5 weeks onwards.



The tuberculous lesions in the respective organs were evaluated as shown in the table below; the total amount is indicated as the mean value.

	Marked Changes	Moderate Changes	Slight Changes
Spleen .....	35	20	10
Lungs .....	30	20	10
Liver .....	25	20	10
Inoculation .....	10	10	10

3. No distinct difference was observable in the reversal of the tuberculin allergy or in the weight increase of the animals in the course of the experiment.

#### EXPERIMENT II

Based on the results of Experiment I, the next experiment was planned as follows:

*Material.* — 30 animals, mean weight 355 g, were used. Before the experiment the animals gave a negative reaction to a Mantoux test with 1.0 mg of Alt-Tuberculin. They were inoculated subcutaneously at the sternum with 0.3 mg of a 7-day culture of *M. tuberculosis* H<sub>37</sub>Rv (the quantity was determined according to the centrifuging method).

The animals were divided into two groups:

Group A: control, no treatment.

Group B: the animals of this group were treated with ACTH, 2 mg subcutaneously every two days.

All the animals were sacrificed and autopsied 4 weeks after the inoculation.

*Results.* — 1. At autopsy and in the mapping of the macroscopic findings, the principles followed were those described for Experiment I. The results are shown in Fig. 2.

As can be seen from Fig. 2, a distinct difference is observable between the animals of the two groups and distinct macroscopic changes can be observed in ACTH-treated animals within 4 weeks of the inoculation.

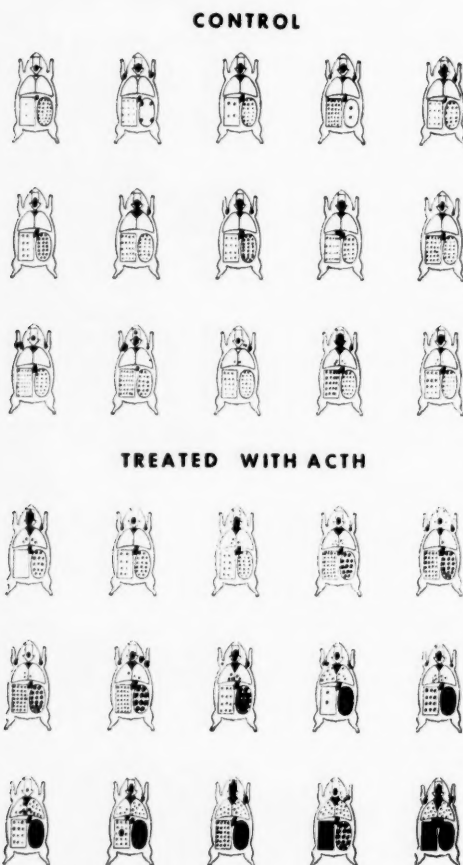


Fig. 2.

2. The results have also been computed according to Feldman's (2,4) principle (cf. Exp. I), and are given in Table 2, where the figures refer to the mean value of the sum of the total lesions per group.

TABLE 2

Group A (control) . . . .	46
Group B (ACTH) . . . .	67

3. No difference in weight increase or reversal of tuberculin allergy was noticeable between the animals of the two groups.

## DISCUSSION

Experiment I showed that distinct macroscopic tuberculous lesions are found in the 3rd—5th week after the TB inoculation of ACTH-treated experimental animals. Experiment II showed these changes after 4 weeks in a larger material.

It is recognized in the literature that ACTH and cortisone aggravate experimental tuberculosis. Jørgensen & Ringsted (3), basing themselves on an investigation by Engbock, Friis & Teillum (1), were able to show macroscopic tuberculous lesions in guinea-pigs treated with cortisone within four weeks; however, because their material was limited they drew no definite conclusions. From our own results we believe that the guinea-pig test for tuberculosis can be shortened to two-thirds of the present time, viz. from 6 to 4 weeks, by treating the experimental animals with ACTH.

In addition, we have suggested the possibility of shortening the diagnostic guinea-pig test for tuberculosis by various vaccines (5). The suggestion is based on thorough experimental studies (6, 7).

## SUMMARY

The authors made two experiments comprising a total of 46 guinea-pigs and showed from them that by treating animals used for the guinea-pig test for tuberculosis with ACTH the duration of the test can be reduced from 6 to 4 weeks.

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## EFFECT OF CHLORPROMAZINE-HIBERNATION ON STAPHYLOCOCCAL INFECTION

### QUANTITATIVE BACTERIAL POPULATION STUDIES

by

O. WASZ-HÖCKERT, A. W. ERIKSSON and T. KOSUNEN

(Received for publication October 11, 1956)

The subject of the present study, the influence of artificial hypothermia on resistance to an acute infection, forms a component part of the host-parasite relationship studies performed in our laboratory. Hibernation is of importance in clinical medicine and research, which has prompted us to report on our investigation.

Our experimental study was performed on mice. The quantitative degree of the infection was measured by a special bacilli enumeration technique. Since the mice became sleepy on hibernation an other control group received barbiturate.

### MATERIAL AND TECHNIQUE

1. *Mice*. — Eighty albino mice (Wiele-strain), weight range 18—30 g, were used.

2. A coagulate positive *Staphylococcus aureus* strain, »Orion», was employed. The inoculation dose was 0.2 ml of a 20-hour broth culture, diluted 1: 2 with physiological saline.

The challenge was performed by injection into one of the tail veins of the mouse.

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<sup>1</sup> Aided by grants from the Sigrid Jusélius Foundation and State Commission for Sciences.

3. 30 mgm/kgm of a 0.1% dilution of *chlorpromazinehydrochloride* (Largactil, M&B) was injected subcutaneously daily for 13 days in the inguinal region. Further studies concerning the effect of chlorpromazine on mice are reported elsewhere (4).

4. *Barbiturate*, a 0.5% dilution of lympinal-natrium (Sodium-phenylaethylberbituric acid), was used in a dosage of 100 mgm/kgm in order to provide a control group animals as somnolent as in the hibernation group. Shortly after the injection the mice behaved as in a typical excitation state, but later their degree of somnolence was comparable with that in the hibernation group.

5. Physiological saline was given to the control group to eliminate possible differences due to stress and fluid balance.

6. The *temperature* of the mice was measured in sigmoideum with a precision thermometer<sup>1</sup> operating on the thermo-couple principle. The range was 16—46 centigrade degrees; 1/10 degree centigrade changes were clearly observed. During the experiments the room temperature was 17—19 centigrades, and the thermometer was specially designed for automatic compensation of any variation in room temperature. The rectal applicator (type RM 4) was always inserted to exactly the same depth in sigmoideum.

The temperature of the mice was measured twice daily, once before the hibernation dose was injected and once 6 hour later. Additional readings were made if needed.

7. The experimental conditions were kept very similar for all the animals (room temperature, water and food pellets ad libitum). Five mice were kept in each cage.

8. The weight of the mice was measured twice, immediately before challenge and after 7 days of the experiment.

9. The bacterial enumeration technique employed was familiar to one of the authors from Cornell University Medical College and is described in detail from the Rockefeller Institution (3). In brief, however, the technique is as follows: The number of bacteria present in the organs at any given time have been determined. Animals from each group are sacrificed, the kidneys removed under sterile conditions, the volume estimated by displacement in a graduated

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<sup>1</sup> Electric universal thermometer Type TE<sub>3</sub>, Electrolab., Copenhagen.

tube and the organs ground with saline in pyrex tubes with Teflon homogenizers. The solution is then diluted as required and inoculated on blood-agar plates. These are incubated at 37°C for 24 hours and the bacilli-colonies counted.

In calculating the results, consideration is paid to the volume of the kidneys, the dilution and the growth of the bacilli. The number of bacteria are then reported in logarithms.

After challenging the mice with staphylococci, the animals were divided into three groups:

1. *Control group*, 14 male and 13 female mice were daily injected subcutaneously with 0.6 ml of physiological saline.

2. *Hibernation group*, 14 male and 13 female mice were daily injected in proportion to bodyweight with 0.1% chlorpromazine solution (0.54–0.90 ml subcutaneously).

3. *Barbiturate group*, 23 male mice were daily injected in proportion to bodyweight with a 0.5% dilution of luminal natrium (0.36–0.60 ml subcutaneously).

Mice were sacrificed by random sampling 1–5, 7, 9, 11 and 13 days after inoculation, respectively.

## RESULTS

The behaviour of the test animals during the course of the experiment was observed. The hibernating mice usually lay down, but showed an interest in their food intake. The degree of somnolence was thus comparable with that in the barbiturate group.

1. *Temperature*. — There was a fall in the temperature curve in all groups. The mean values will be seen from Fig. 1, but for the hibernation group the lowest and highest temperature means are plotted.

2. *Weight*. — A weight loss was stated in all three groups, 25% for the saline-injected control group, 18% for the chlorpromazine and 10% for the barbiturate, taking the mean weight value at 7 days.

3. *Deaths*. — In the control and hibernation groups there were two deaths in the entire 13 day period. In the barbiturate group, however, 9 mice out of 24 died.

# TEMPERATURE IN CENTIGRADE

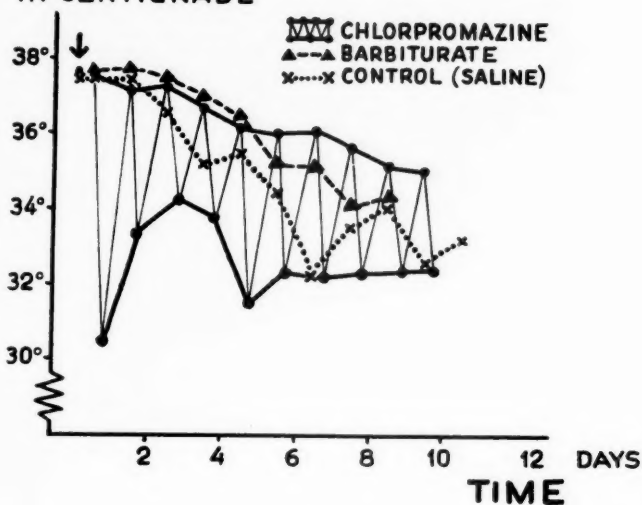


Fig. 1.

4. The staphylococci population growth in the kidneys as measured by the quantitative bacterial enumeration technique is shown in Figure 2.

As the graph of the results indicates, the trend line for the control group shows the bacterial multiplication.

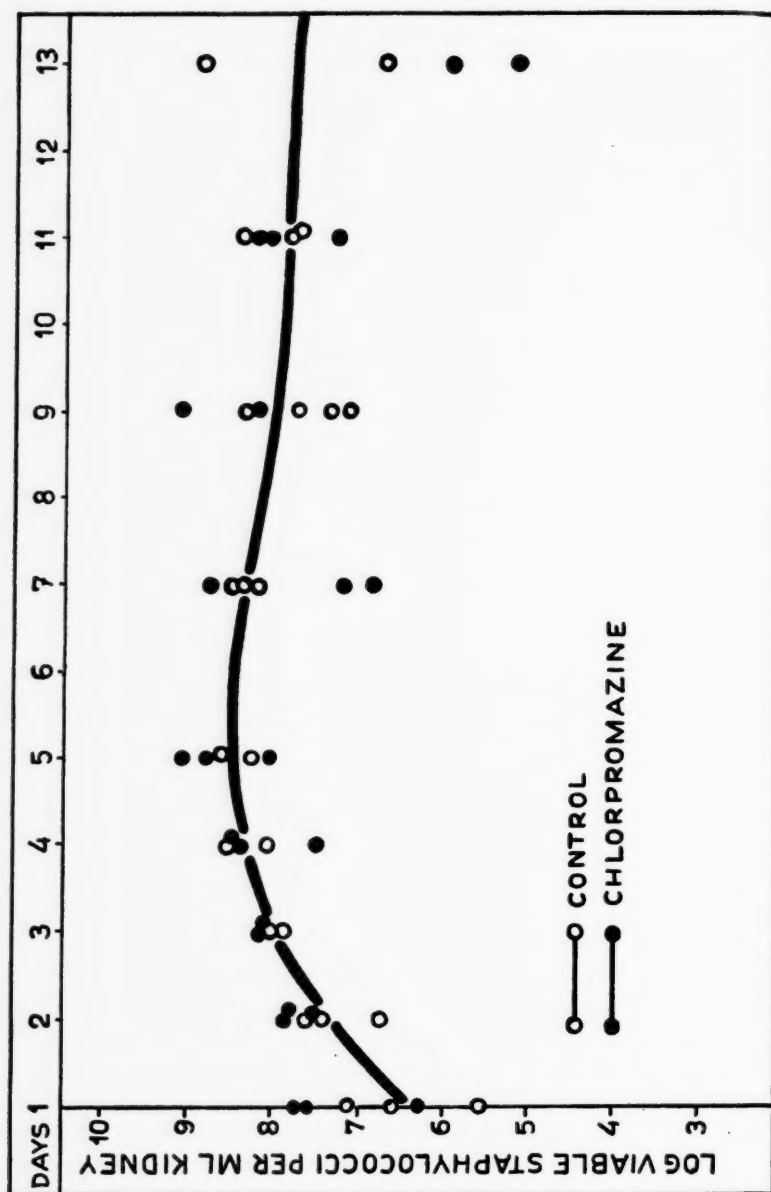
The staphylococci values for the kidneys of the hibernation group are not significantly different from those of the control group. The range however is broader.

In the barbiturate group, too, the values remained within insignificant limits. Regarding the deaths more detailed studies are to be made of the effect of various sedatives on the course of infection a question of considerable interest.

## DISCUSSION

Our results show that chlorpromazine-induced experimental hibernation in mice did not affect the resistance of the host to an acute virulent staphylococci infection. From studies where hypothermia was induced by keeping rabbits in either cold baths or





a refrigerator (1), thus maintaining a rectal temperature around 31–33°C, it was reported (2) that there was «an almost critical fever level below which the animal's temperature may not be permitted to range during the early period of infection without imperiling survival». The pneumococci infection used in these experiments caused overwhelming bacteremia when a virulent strain was used and the rabbits hypothermed.

Although our studies are to be continued from the theoretical point of view, on the lines of hibernation, hyperpyrexia and barbiturates, the results already attained might be used to stress the fact that chlorpromazine-induced hypothermia does not seem to influence the resistance to infections present under hibernation. This may also have valuable clinical applications.

#### SUMMARY

Mice inoculated with a staphylococci infection where hibernated with chlorpromazine for 13 days. The bacterial multiplication was determined quantitatively from the kidneys. The difference from a control group was not significant. Mice treated with barbiturate were used as a second control group.

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TRANSMISSION OF ANTIBODIES FROM MOTHER TO FETUS  
A STUDY OF THE DIPHTHERIA ANTITOXIN LEVEL IN THE NEWBORN  
WITH OESOPHAGEAL ATRESIA

by

O. WASZ-HÖCKERT, O. WAGER, TUULA HAUTALA, and O. WIDHOLM

(Received for publication October 24, 1956)

The question of the mechanism of antibody transfer from mother to fetus has not been adequately resolved. It has been shown in extensive experimental studies carried out on rabbits by Brambell and coworkers that transfer of antibodies from mother to fetus takes place by way of the uterine lumen and not via the placenta (1) and that antibodies are absorbed in the gut of young animals (2). The route of the antibody transfer would be by swallowing the amniotic fluid and, from the fetal gut, through absorption into the fetal circulation (3). A similar transfer for man has been doubted by Smith (4), since the present knowledge indicates that antibodies swallowed by the human infant soon after birth are not absorbed to a significant degree, and that antibodies as proteins are more likely to be destroyed.

Our intention is to contribute to this discussion simply by investigating whether or not diphtheria antitoxin is present in the circulation of newborn with oesophageal atresia who have obviously been unable during fetal life to swallow amniotic fluid.

MATERIAL AND METHODS

Six newborn children operated upon for *congenital oesophageal atresia* at the Children's Clinic, Surgical Department, represent the series of our

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investigation. The birthweight of these newborn ranged from 2150 to 3700 g. One of them deserves special attention (3893/56), since both anal and oesophageal atresia were present in this case, and furthermore, there was no fistula between the oesophagus and trachea. This finding was confirmed both at operation (M. Sulamaa, M.D.) and by autopsy (L. Hjelt, M.D.). — All the other children had both oesophageal atresia and tracheo-oesophageal fistula. None of them received any blood, plasma or fluid therapy before a 5 ml bloodsample had been taken; it was taken not later than 24 hour after birth. Sera from the mothers were collected with the cooperation of the physicians who performed the deliveries. A control series of nine mother-and-child pairs from the Women's Clinic was also investigated. The children were perfectly healthy.

*Assay of Antitoxin.* — The technique of the intracutaneous rabbit test was followed (5) with the exception that, for reasons of economy, guinea pigs were used instead of rabbits.

The standard antitoxin used was obtained from the Danish State Serum Institute (lot DI 45 & 46). The same lot of diphtheria toxin was used for all the assays. This toxin has been described in an earlier paper (6), in which additional information on the assay technique also is given.

#### RESULTS AND CONCLUSIONS

The levels of antitoxin in the sera of mothers and their newborn children are shown in the following table.

TABLE

Case No.		Level of Diphtheria Antitoxin (units pro ml serum)	
Cases of Atresia		Mother	Child
	3301/56	0.01	0.01
	3224/56	0.3	0.3
	3893/56	1—2	1—2
	3960/56	0.2—0.5	0.2—0.5
	3645/56	< 0.01	< 0.01
	1970/56	< 0.01	< 0.01
Normal Controls		0.3—1.0	0.5—0.8
		0.1	0.1—1.0
		0.1—1.0	0.1—1.0
		< 0.01	< 0.01
		< 0.01	< 0.01
		< 0.01	< 0.01
		0.2—0.5	0.5—0.8
		0.1—0.2	0.2—0.5
		0.2—0.5	0.2—0.5

The results shown in the above table permit the following conclusions:

In the sera of newborn children an antitoxin level comparable to that of the mother is present. In the case of children with oesophageal atresia, with or without an oesophago-tracheal fistula and with additional anal-atresia the antitoxin level is similarly comparable to that of the mother.

These results obtained in our investigation show clearly that in man the swallowing of the amniotic fluid by the fetus is not, as has been suggested from animal experiments, the only if any route of antibody transfer.

Our study does not give an answer to the question whether amniotic fluid aspirated into the lungs during the fetal life could be a route of antibody transfer from mother to fetus.

#### SUMMARY

The level of diphtheria antitoxin was measured in the circulation of newborn infants with oesophageal atresia, and of their mothers.

In all cases the level of antitoxin in the sera of the children was comparable to that of the mother.

Thus, the swallowing of the amniotic fluid by fetus is not, as has been suggested from animal experiments, the only if any route of antibody transfer from mother to fetus.

*Acknowledgment.* — The authors wish to thank Joseph Dancis, M.D., for fruitful discussions on this subject.

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## IS THE SEX RATIO BETWEEN BOYS AND GIRLS CORRELATED TO THE SEX OF PRECEDENT CHILDREN?<sup>1</sup>

by

K. O. RENKONEN

(Received for publication October 2, 1956)

Figures between 105 or 106 boys to 100 girls among live births are considered normal. Among stillbirths the ratio is considerably higher (6). According to the birth order the sex ratio has a slight tendency to decrease, it drops from 106.3 by the first child to 105.3 by the fourth child (2).

Since the discovery of rhesus blood groups the idea of incompatibility between mother and child has steadily won in importance. Considering the high mortality of male children in utero and during the first period of life the question arises is there also an antagonism due to immunisation among the hostility factors.

Statistical information upon the sex ratio when considering the sex of precedent children seemed desirable, but was not available for us. Thus the aim of this paper was to give preliminary figures on the subject. — Data concerning the proportion of unisexual sibships are collected elsewhere and discussed by previous workers (1, 3, 4, 5, 7, 8).

Our figures are collected partly from population records kept by parishes, partly from current biographies and from maternity stations. We have eliminated all families with twins, all families with illegitimate children or children from a previous marriage of the wife.

The following table gives the present size and grouping of the figures collected:

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<sup>1</sup> Supported by grants from Sigrid Jusélius Foundation and from State Commission on Natural Science.

TABLE 1  
THE DISTRIBUTION OF CHILDREN ACCORDING TO THE BIRTH ORDER OF BOYS AND GIRLS

	No. of Fam- ilies	Sex Ratio by Second Child		No. of Fam- ilies	Sex Ratio by Third Child		No. of Fam- ilies	Sex Ratio by Fourth Child
boy-boy	8,058	105.4	boy-boy-boy	2,511	103.5	boy-boy-boy-boy	742	109.6
			boy-boy-girl	2,427		boy-boy-boy-girl	677	
boy-girl	7,642	105.4	boy-girl-boy	2,166	94.7	boy-boy-girl-boy	671	96.5
			boy-girl-girl	2,288		boy-boy-girl-girl	695	
girl-boy	7,687	98.2	girl-boy-boy	2,278	105.6	boy-girl-boy-boy	595	92.0
			girl-boy-girl	2,157		boy-girl-boy-girl	647	
girl-girl	7,828	98.2	girl-girl-boy	2,356	97.9	girl-girl-boy-boy	677	103.4
			girl-girl-girl	2,406		girl-girl-girl-girl	655	
Total	31,215			18,589			10,530	



The figures are not great enough for correct indication of the size of different groups, but something can, however, be deduced.

In two children families (31,215), the sex ratio was  $105.4 \pm 1.6$  if the first child was a boy, but only  $98.2 \pm 1.6$  if it was a girl. Statistically the difference  $7.2 \pm 2.3$  is significant \*. Thus there is evidence that the sex ratio is correlated to the sex of the previous child. There seems to be a tendency to duplicate the sex of a previous child.

In three children families (18,589) we obtained the following figures, if we group the series according the sex of the second and third child and neglect the sex of the first.

TABLE 2

THE DISTRIBUTION OF CHILDREN BY SEX OF THE SECOND AND THIRD CHILD

	No. of Families	Sex Ratio of the Third Child
Boy-boy .....	4,789	104.5
boy-girl .....	4,584	
girl-boy .....	4,522	96.3
girl-girl .....	4,694	

The difference in sex ratios  $8.2 \pm 3.0$  is still significant\* and similar to that met by the second child. The tendency to duplicate the sex of the second child by the third is marked.

If we group the four children families (10,530) in an analogous way and neglect the sex of the two first children, we obtain the figures seen in Table 3.

TABLE 3

THE DISTRIBUTION OF CHILDREN BY SEX OF THE THIRD AND FOURTH CHILD

	No. of Families	Sex Ratio of the Fourth Child
Boy-boy .....	2,673	103.5
boy-boy .....	2,583	
girl-boy .....	2,629	99.4
girl-girl .....	2,645	

\* The probability is over 99 % but less than 99.9 %. The statistical analysis performed by E. K. Kaila, Ph. D., is based on following calculations. The probability of a boy is  $p$  and the probability of a girl is  $q = 1 - p$ . The number of cases is  $n$ . The standard deviation of  $p$  and  $q$  is thus  $\sqrt{\frac{pq}{n}}$ . When approxim-

ately  $p \approx q \approx \frac{1}{2}$ , the standard deviation of  $\frac{p}{q}$  is  $\sqrt{\frac{p}{nq^3}} \approx \frac{2}{\sqrt{n}}$ .

The difference in sex ratios  $4.1 \pm 4.0$  is not significant but may be of the same quality as with the second and third child. Other factors might also influence the sex of the fourth child, not only the duplication tendency. In fact duplication is visible only in five of the eight pairs of four children families (Table 1).

Considering the whole series<sup>1</sup> there is a highly significant tendency (probability over 99.9%) for the child to copy the sex of the precedent.

The existence of hereditary factors is frequently suggested. The discussion goes around the problem whether there is a statistically significant excess of unisexual sibships. This is true in a large Saxonian set of data collected by Geissler (3) and in a series (round 6,000 families) collected by Bernstein (1). Two other authorities (5 and 7), on the basis of their series comprising 999 and 1,269 families, respectively, found the difference to be insignificant.

In our series there is an overweight of unisexual sibships.

TABLE 4  
OCCURRENCE OF UNISEXUAL SIBSHIPS

		Sibships	Ratio Unisexual/ Bisexual Sibships
Two-child families:	unisexual (types mm and ff)	15,886	$103.6 \pm 1.1$
	bisexual (types mf and fm)	15,329	
Three-child families:	unisexual (types mmm and fff)	4,917	$102.8 \pm 2.0$
	bisexual (types mmf and ffm)	4,783	
Four-child families:	unisexual (types mmmm and ffff)	1,476	$110.9 \pm 3.8$
	bisexual (types mmmf and ffmf)	1,331	

The difference in favor of unisexual sibships is statistically significant in the two- and four-child families. It might be due to the tendency of the second and third child to copy the sex of the precedent. It might also be due to hereditary factors. We don't think that the duplication tendency is based on hereditary factors only, because if we compare the sex ratios  $94.7 \pm 3.0$  and  $105.6 \pm 3.0$  of the third child in the groups beginning either with the boy-girl

<sup>1</sup> Boy-boy group 15,520 (= 8,058 + 4,789 + 2,673), boy-girl group 14,809, girl-boy group 14,838 and girl-girl group 15,167. Sex ratios  $104.8 \pm 1.2$  and  $97.8 \pm 1.2$  respectively. Difference  $7.0 \pm 1.7$ .

or girl-boy sibships (Table 1), we meet also here the duplication tendency. Statistically the difference  $10.9 \pm 4.2$  is very close to a significance level of 99%. This difference cannot be caused by hereditary factors. Accordingly we are inclined to accept an existence of hereditary factors mainly on the basis of the figures offered by four-child families as the sex duplication tendency with the fourth child was weak.

The problem of a possible immunisation and an incompatibility on that basis could be discussed on the basis of the figures in Table 1 (sex ratios 92.0 and 111.8 after boy-girl-boy and girl-boy-girl sibships respectively), but the size of the figures do not allow definite conclusions. — The four children families are not numerous enough even for a closer analysis of the interesting assymetry of their sex ratios as seen from in Table 1.

#### SUMMARY

Variations in sex ratios are studied by analysing a family series consisting of 31,215 families with two children, 18,589 families with three children and 10,530 families with four children.

The sex ratios in the second, third and fourth birth order seem to be correlated to the sex of the precedent child. There is a highly significant tendency to duplicate the sex of the precedent child. This tendency is most pronounced by the second and third child, less by the fourth. An influence of hereditary factors cannot be denied. The best evidence for their existence is offered by the figures of four children families.

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